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DIFFERENTIAL EFFECTS OF *BACILLUS* SPECIES-FERMENTED PRODUCTS ON ANTIBIOTIC RESISTOME AND VIRULENCE FACTOR GENE COMPOSITION IN THE CECAL DIGESTA OF BROILERS*

Felix Shih-Hsiang Hsiao, Yeong-Hsiang Cheng, Hsiu-Wei Chen, Yu-Hsiang Yu♦

Department of Biotechnology and Animal Science, National Ilan University, Yilan 26047, Taiwan
♦Corresponding author: yuyh@niu.edu.tw

Abstract

This study investigated the differential effects of *Bacillus subtilis*- and *Bacillus licheniformis*-fermented products (defined as SFP and LFPs, respectively) on microbial antibiotic resistance gene (ARG) and virulence factor gene (VFG) composition in the cecal digesta of 35-day-old broilers by using metagenomic sequencing. First, 160 1-day-old unsexed Arbor Acres broiler chicks were randomly allocated to four treatment groups: basal diet (CON group); basal diet + enramycin (10 mg/kg, ENM group); basal diet + SFPs (10^8 colony-forming units (CFU) of *B. subtilis* spores/kg, SFP group); and basal diet + LFPs (10^8 CFU of *B. licheniformis* spores/kg, LFP group). Principal coordinate analysis of ARG and VFG composition indicated distinct clustering among the cecal samples of the groups. At the antibiotic resistance class level, LFP treatment increased the expression of peptide resistance genes and decreased the expression of aminocoumarin resistance genes compared with the other groups. Compared with the other groups, LFP treatment promoted *bcrA*, *ugd*, and *efrB* expression but suppressed *parY* expression, whereas SFP treatment inhibited *efrA* expression. The abundance of the peptide resistance gene *bcrA* in *Lachnoclostridium* species was higher in the CON and LFP groups than in the ENM and SFP groups, whereas the abundance of the peptide resistance gene *rpoB2* in *Bacteroides* species was lower in the ENM and LFP groups than in the SFP group. No specific VFGs were regulated only by SFPs or LFPs. SFP and LFP treatment inhibited *clpC* expression compared with the other groups. *clpC* abundance in *Bacteroides* species was lower in the LFP group than in the CON group, whereas its abundance in *Faecalibacterium* species was lower in the SFP group than in the CON and ENM groups. These results demonstrated that SFPs and LFPs differentially regulate microbial ARG and VFG composition in the cecal digesta of broilers. LFP supplementation modulated more antibiotic resistance classes and ARGs than did SFP supplementation.

Key words: ***Bacillus subtilis*, *Bacillus licheniformis*, broiler, antibiotic resistance gene, virulence factor gene**

Antimicrobial resistance has become a global public health concern due to the overuse of antibiotics in food-producing animals (Winglee et al., 2017). Antibiotics have been commonly used as growth promoters in poultry farms, thereby creating constant selective pressure for antimicrobial resistance to microorganisms (Xiong et al., 2018; De Cesare et al., 2022). Antibiotic resistance genes (ARGs) derived from poultry farms eventually enter the human food chain (Su et al., 2015). Pathogenic bacteria possess various virulence factor genes (VFGs) that allow them to cause infection and survive in hosts (Wu et al., 2008). Targeting the main VFGs of pathogens is considered a new therapeutic approach (Gavrish et al., 2014; Choules et al., 2019). The European Union has banned the routine use of antibiotics as growth promoters in food-producing animals, causing researchers to explore novel alternatives for poultry farming, such as probiotics, prebiotics, and phytobiotics (Aljumaah et al., 2020; Hussein et al., 2020; Hafeez et al., 2020; Shah et al., 2020). However, their effects on ARG and VFG composition in the genome of gut microbiota remain unclear.

Bacillus species are one of the most common bacterial species used in commercial probiotic products for poultry production (Ramlucken et al., 2020); in particular, they have been used to prevent pathogen infection and improve the growth performance of broilers (Ramlucken et al., 2020). Submerged fermentation has been commonly used for probiotics production. Recently, the use of solid-state fermentation has been increasing for the production of *Bacillus* species-based feed and feed additives due to its environmental sustainability (Yang et al., 2021). We previously demonstrated that *Bacillus subtilis*-fermented products (SFPs) produced using solid-state fermentation improved the growth performance and reduced the gut inflammation of broilers in response to inflammatory challenges (Chen and Yu, 2021, 2022 a).

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SFPs also reshape the cecal microbiota community of broilers by increasing the abundance of short-chain fatty acid–producing bacteria and reducing mucin-degrading bacteria in cecal digesta (Chen and Yu, 2022 a). *Bacillus licheniformis*-fermented products (LFPs) produced using solid-state fermentation can enhance the growth performance and prevent coccidiosis in broilers (Chen and Yu, 2020; Cheng et al., 2021). LFPs can modulate gut microbial composition by increasing *Lactobacillus* abundance in broilers (Chen and Yu, 2020, 2022 a; Cheng et al., 2021).

Changes in gut microbiota composition accompany changes in the abundance of bacterial hosts carrying specific ARGs and VFGs in broiler gut microbiota (Xiong et al., 2018; Chen and Yu, 2022 b). *B. subtilis* and *B. licheniformis* have distinct antimicrobial mechanisms (Tran et al., 2022). We previously demonstrated that SFPs and LFPs differentially modulate the cecal microbial community of broilers (Chen and Yu, 2023). However, the differential effect of these two solid-state fermented products on ARG and VFG diversity and distribution from the gut of broilers, as determined using metagenomic sequencing, remains unclear.

We hypothesized that SFPs and LFPs would differentially regulate ARG and VFG composition in the gut microbiota of broilers. In this study, we used metagenomic sequencing to evaluate the effect of *Bacillus* species-fermented products on antibiotic resistome and VFG composition in the gut microbiota of broilers. On the basis of our previous findings that SFPs and LFPs differentially regulate broiler growth performance and gut microbiota (Chen and Yu, 2023), we examined the ARG and VFG composition in the cecal digesta of broilers in the present study.

Material and methods

Experimental design

This research was approved by the Institutional Animal Care and Use Committee of National Ilan University (109-26). The detailed preparation and composition of SFPs and LFPs were described previously (Chen and Yu, 2023). We randomly allocated 160 1-day-old unsexed Arbor Acres broiler chicks to four treatment groups, with eight replicates containing five chicks per replicate. The treatment groups were as follows: the control group (CON), which was fed a corn–soybean-based diet (basal diet); the ENM group, which was fed the basal diet supplemented with enramycin (10 mg/kg); the SFP group, which was fed the basal diet supplemented with SFPs (10^8 colony-forming units (CFU) *B. subtilis* spore/g of feed); and the LFP group, which was fed the basal diet supplemented with LFPs (10^8 CFU *B. licheniformis* spore/g of feed). The basal experimental diets were formulated to meet all of the minimum nutrient requirements of the birds, as determined by the National Research Council (1994). The experiment lasted 35 days and comprised two feeding phases: starter (1–14 days) and grower (15–35 days). The broilers were housed in stainless steel cages, given *ad libitum* access to feed and water, and subjected to a 20-h light–4-h dark cycle throughout the experiment. The ambient temperature on Days 1–3 was maintained at 33°C and was gradually decreased by 1–2°C/day to 24°C, which was maintained until the end of the study. The broilers were vaccinated against Newcastle disease virus and infectious bronchitis virus through nose drops on Days 4 and 14.

Microbial genomic DNA isolation

On Day 35, the broilers were euthanized through carbon dioxide inhalation, and the cecal digesta of two broilers per replicate were freshly collected and pooled. Four replicates (8 birds/treatment, n = 4) were used for metagenomic analysis. The microbial genomic DNA of the cecal digesta was extracted using a ZymoBIOMICS DNA Miniprep Kit (Zymo Research, Irvine, CA, USA), following the manufacturer's instructions.

Library construction, metagenomic sequencing, and annotation

The library was prepared using an Illumina Nextera XTLibrary Preparation Kit (Illumina, San Diego, CA, USA), and sequencing was performed on an Illumina NovaSeq 6000 platform (Illumina) to generate 150-bp paired-end reads. The sequenced raw reads were quality-processed using Trimmomatic (version 0.38) to trim sequence regions where base quality fell below Q20. Reads with a minimum sequence length of 100 bp were removed. Chicken, human, maize, soybean, wheat, and fish sequencing reads were removed using Bowtie2 (version 2.3.4.1) to increase the proportion of microbial sequences. Trimmed, screened, and paired-end Illumina reads were assembled using MEGAHIT (version 1.1.3) with default settings. The assembled contigs were filtered to a minimum length of 500 bp. Gene prediction and annotation were performed with Prodigal (version 2.6.3). Predicted genes with >100 amino acid sequences were selected for annotation. CD-HIT (version 4.6.6) was used to cluster genes and sequence redundancy. All sequencing reads were aligned back to the genes using BWA (version 0.7.17-r1188). The number of mapped reads was calculated using SAMtools (version 1.8). The coverage for all contigs was calculated using jgi_summarize_bam_contig_depths in MetaBat (version 2.12.1). ARG profiling was performed by mapping the amino acid sequences obtained from Prodigal against the Comprehensive Antibiotic Resistance Database by using DIAMOND (version 0.9.22.123). The amino acid sequences of the protein encoded by the gene were aligned in the Virulence Factor Database in DIAMOND. Taxonomic assignments were performed by aligning the protein sequence to NCBI's NR database by using DIAMOND. ARG and VFG diversity was estimated using alpha diversity (richness and evenness) with MicrobiomeAnalyst (Dhariwal et al., 2017). To analyze the overall differences in bacterial

ARG and VFG structures, principal coordinate analysis (PCoA) and heatmap was calculated and plotted using R ggplot2 package (version 3.2.0) and R pheatmap package (version 3.6.3), respectively.

Statistical analysis

Individual cages were considered replicates, defined as experimental units. The Shapiro–Wilk test was used for testing data distribution normality. For the normally distributed data, a one-way analysis of variance including the Tukey's honestly significant difference post hoc test was applied. For the non-normally distributed data, a Kruskal–Wallis test with Dunn's pairwise test was used. The indexes are expressed as means with standard error of mean. All P values were adjusted using the Benjamini and Hochberg method, and statistical significance was indicated by $P<0.05$. The graphs were designed using GraphPad Prism (GraphPad Software, San Diego, CA, USA). PCoA based on Bray–Curtis dissimilarity metrics of the microbial ARG and VFG structure was used to calculate the distance between samples. The Bray–Curtis dissimilarity metrics is widely used to quantify the compositional dissimilarity between samples.

Results

Effect of SFPs and LFPs on the cecal antibiotic resistome of broilers

Figure 1 presents the alpha diversity of antibiotic resistance classes in the cecal digesta of broilers. The richness of macrolide resistance genes was lower in the SFP group than in the CON group (Figure 1 A). By contrast, the richness of glycopeptide resistance genes was lower in the LFP group than in the CON group (Figure 1 A). The diversity of peptide and aminocoumarin resistance genes was higher in the LFP groups than in the ENM and SFP groups (Figure 1 B). The diversity of macrolide resistance genes was higher in the ENM and LFP groups than in the SFP group (Figure 1 B), whereas that of the lincosamide resistance genes was lower in the ENM group than in the CON and SFP groups (Figure 1 B). PCoA exhibited a clear separation of the antibiotic resistance class composition among the groups (Figure 2 A). Figure 2B presents a heatmap showing the 20 most abundant antibiotic resistance classes in the cecal digesta of broilers. Specific antibiotic resistance classes, such as phenicols, aminocoumarins, and lincosamides, were identified in the ENM group. Mupirocin and nitroimidazole resistance genes were identified in the SFP group, whereas peptide and rifamycin resistance genes were identified in the LFP group. Both SFP and LFP groups had some genes in similar antibiotic resistance classes, such as tetracyclines and sulfonamides. The effects of SFPs and LFPs on antibiotic resistance classes in the cecal digesta of broilers are summarized in Table 1. The LFP group exhibited the highest peptide resistance gene abundance. Macrolide resistance gene abundance was higher in the CON group than in the SFP group. The abundance of aminocoumarin and lincosamide resistance genes was higher in the ENM group than in the CON and LFP groups. The abundance of the aminoglycoside resistance gene was higher in the CON and LFP groups than in the SFP group. The abundance of tetracycline resistance genes was higher in the SFP and LFP groups than in the ENM group. The abundance of mupirocin and nitroimidazole resistance genes was higher in the SFP group than in the ENM group. The abundance of phenicol resistance genes was higher in the ENM and SFP groups than in the CON group. The abundance of oxazolidinone resistance genes was higher in the ENM group than in the CON group. The abundance of monobactam resistance genes was higher in the CON group than in the ENM and LFP groups. No significant between-group differences were observed in the alpha diversity of the total ARGs (Figure 2 C). PCoA revealed a clear separation of ARG composition among the groups (Figure 2 D). Figure 2 E presents a heat map showing the 35 most abundant ARGs in the cecal digesta of broilers. Specific ARGs, such as *LlmA*, *novA*, and *cat,* were identified in the ENM group, whereas ARGs, such as *kdpE*, *tetB(60)*, *ugd*, *vanRG*, *efrB*, and *bcrA,* were identified in the LFP group. Some ARGs, such as *TaeA*, *cmeB*, *tet37*, and *mdsB,* specifically presented in the CON and SFP groups. The effects of SFPs and LFPs on ARGs in the cecal digesta of broilers are summarized in Table 2. The abundance levels of *bcrA*, *ugd*, and *efrB* were highest in the LFP group, and *efrA* abundance was lowest in the SFP group. *LlmA* and *novA* abundance was highest in the ENM group, but *parY* abundance was lowest in the LFP group. *rpoB2* abundance was highest in the SFP group, but *ANT(6)-Ib* abundance was lower in the ENM and SFP groups than in the other groups. *vanRI* abundance was lower in the SFP group than in the CON and LFP groups, but *tet32* abundance was higher in the SFP group than in the LFP group. *optrA* abundance was higher in the ENM group than in the CON group, but *msbA* abundance was lower in the ENM group than in the SFP group. No significant between-group differences were noted in the total number of ARGs (Table 2).

Effect of SFPs and LFPs on the taxonomic assignment of ARGs

Figure 3 presents the four most abundant genera with antibiotic resistance classes and the most abundantly assigned ARGs in the cecal digesta of broilers. In *Lachnoclostridium* and *Bacteroides* species, ARGs from peptide antibiotic resistance classes were predominant. The glycopeptide antibiotic resistance class was the most abundant in *Blautia* species, whereas ARGs from the macrolide antibiotic resistance class were the most abundant in *Clostridium* species. The peptide resistance genes *bcrA* and *rpoB2* were predominantly

associated with *Lachnoclostridium* and *Bacteroides* species, respectively. The macrolide resistance genes *efrA* and *efrB* were the most abundant in *Blautia* and *Bacteroides* species, respectively. The effects of SFPs and LFPs on the dominant genera with the dominant assigned antibiotic resistance class and gene in the cecal digesta of broilers are summarized in Table 3. At the antibiotic resistance class level, the abundance of peptide resistance genes in *Lachnoclostridium* species was lower in the SFP group than in the CON and LFP groups, whereas the abundance of glycopeptide resistance genes in *Blautia* species was higher in the SFP group than in the CON and ENM groups. The abundance of peptide resistance genes in the *Bacteroides* species was higher in the LFP group than in the CON and ENM groups. At the individual ARG level, *bcrA* abundance in *Lachnoclostridium* species was higher in the CON and LFP groups than in the ENM and SFP groups. *efrA* abundance in *Blautia* species was lower in the CON and LFP groups than in the ENM group. *efrB* abundance in *Clostridium* species was higher in the LFP group than in the CON group, whereas *rpoB2* abundance in *Bacteroides* species was higher in the SFP group than in the ENM and LFP groups.

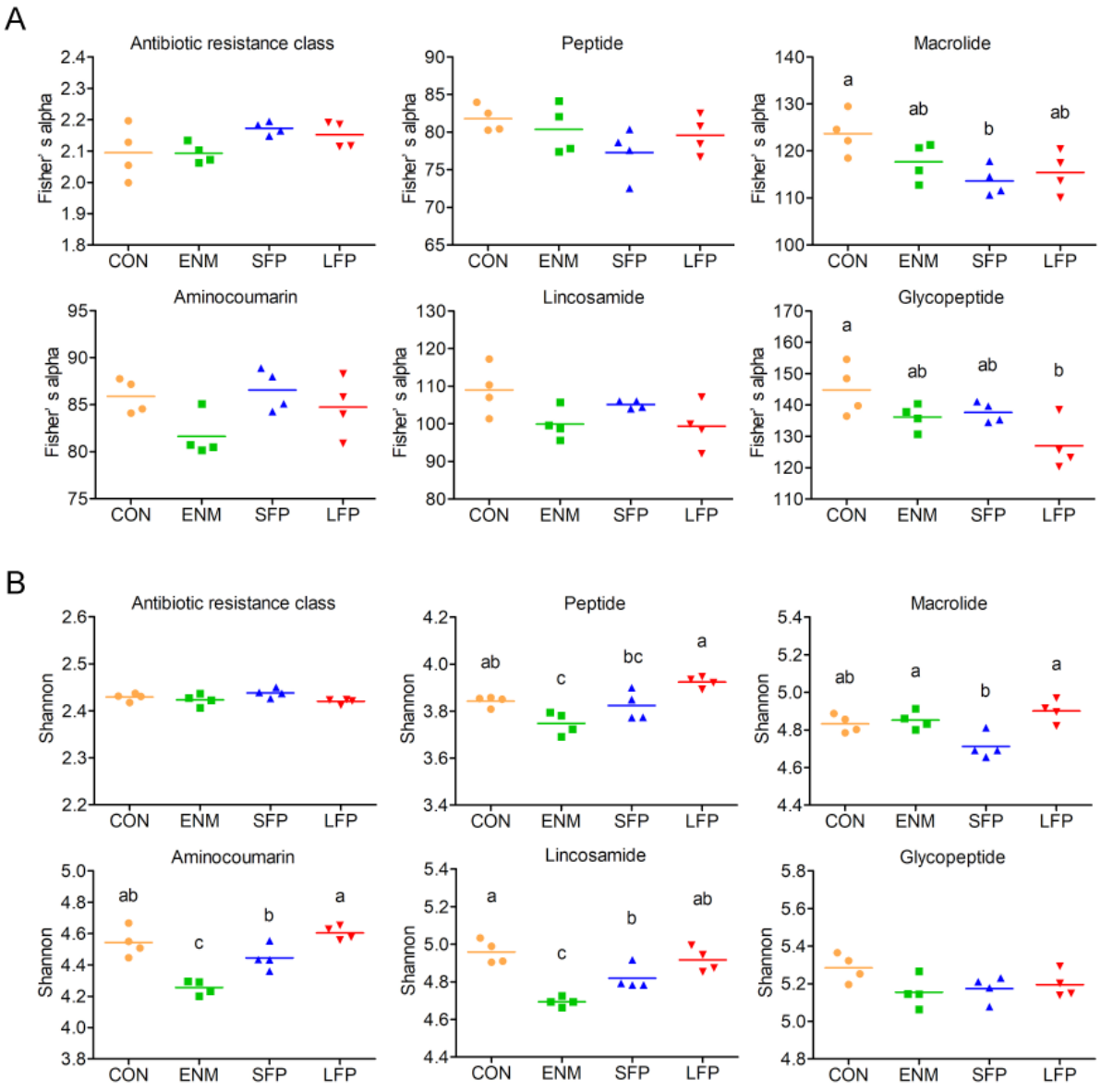

Figure 1. Alpha diversity of different antibiotic resistance classes measured using antibiotic resistance genes in the cecal digesta of broilers. Scatter plot of the alpha diversity of antibiotic resistance classes in the CON (basal diet), ENM (basal diet plus 10 mg/kg enramycin), SFP (basal diet plus *B. subtilis*-fermented products), and LFP (basal diet plus *B. licheniformis*-fermented products) groups. Each bar represents the mean ± SEM (n = 4). Different superscripts indicate significant differences between the groups

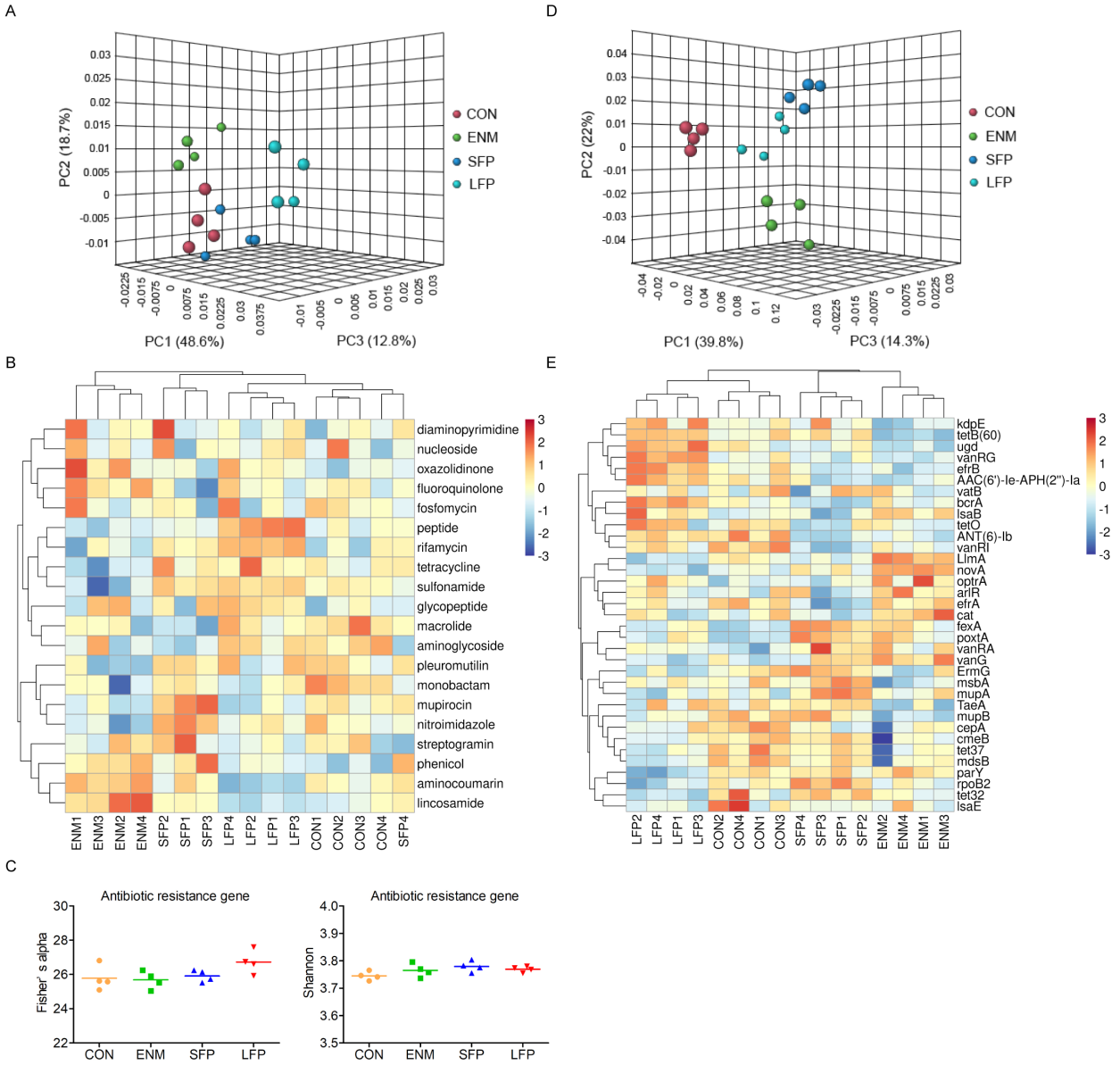

Figure 2. Comparison of the antibiotic resistome in cecal digesta through advanced analysis. (A) Principal coordinate analysis of the antibiotic resistance classes of basal diet in CON (basal diet), ENM (basal diet plus 10 mg/kg enramycin), SFP (basal diet plus *B. subtilis*-fermented products), and LFP (basal diet plus *B. licheniformis*-fermented products) (n = 4) groups. (B) Heatmap of antibiotic resistance class abundance from cecal digesta. The abundance distribution of 20 dominant antibiotic resistance classes (Y-axis) across all samples (X-axis) is displayed (n = 4). (C) Scatter plot of the alpha diversity of ARGs in the cecal digesta of CON (basal diet), ENM (basal diet plus 10 mg/kg enramycin), BFP (basal diet plus *B. subtilis*-fermented products), and LFP (basal diet plus *B. licheniformis*-fermented products). Each bar represents the mean ± SEM (n = 4) groups. (D) Principal coordinate analysis of the individual antibiotic resistance genes in the basal diet of the CON, ENM, SFP, and LFP groups (n = 4). (E) Heatmap of individual antibiotic resistance gene abundance from cecal digesta. The abundance distribution of 35 dominant antibiotic resistance genes (Y-axis) across all samples (X-axis) is displayed (n = 4)

Table 1. Effect of *Bacillus* species-fermented products on antibiotic resistance class composition in the cecal digesta of broilers

	Relative abundance (%)				SEM	P value
	CON[1]	ENM	SFP	LFP		
1	2	3	4	5	6	7
Peptide	20.54 b	20.16 b	20.53 b	21.73 a	0.16	<0.001
Macrolide	12.49 a	12.00 ab	11.76 b	12.28 ab	0.10	0.033
Aminocoumarin	10.95 b	11.68 a	11.11 ab	9.95 c	0.17	<0.001
Lincosamide	9.59 bc	10.46 a	9.87 b	9.34 c	0.12	<0.001
Glycopeptide	9.52	9.80	9.67	10.00	0.09	0.289
Aminoglycoside	9.03 a	8.62 ab	8.28 b	8.96 a	0.10	0.020
Tetracycline	8.32 ab	7.97 b	8.50 a	8.51 a	0.08	0.020

Table 1 – contd.

1	2	3	4	5	6	7
Mupirocin	3.51 ab	3.29 b	3.78 a	3.26 b	0.06	<0.001
Phenicol	3.03 b	3.57 a	3.64 a	3.23 ab	0.08	0.003
Pleuromutilin	2.88	2.69	2.93	2.87	0.03	0.058
Streptogramin	2.21	2.21	2.22	2.16	0.02	0.782
Oxazolidinone	2.13 b	2.30 a	2.14 ab	2.24 ab	0.03	0.034
Monobactam	1.95 a	1.43 b	1.65 ab	1.52 b	0.07	0.013
Fluoroquinolone	1.88	2.07	1.75	1.90	0.04	0.058
Nitroimidazole	1.76 ab	1.54 b	1.90 a	1.73 ab	0.04	0.010

[1]CON = Basal diet; ENM = Basal diet plus enramycin; SFP = Basal diet plus SFPs; LFP = Basal diet plus LFPs.
a–c – mean values in the row without common letters are significantly different (P≤0.05).

Table 2. Effect of *Bacillus* species-fermented products on antibiotic resistance gene composition in the cecal digesta of broilers

	Relative abundance (%)				SEM	P value
	CON[1]	ENM	SFP	LFP		
bcrA	10.42 b	10.36 b	9.81 c	11.26 a	0.14	<0.001
parY	7.47 a	7.54 a	7.44 a	6.68 b	0.10	<0.001
rpoB2	5.45 b	5.29 bc	5.83 a	5.04 c	0.08	<0.001
LlmA	5.21 b	5.54 a	5.03 bc	4.90 c	0.07	<0.001
efrA	4.72 a	4.66 a	3.97 b	4.39 a	0.09	<0.001
ANT(6)-Ib	4.51 a	4.01 b	3.91 b	4.42 a	0.08	<0.001
ugd	4.22 b	3.88 b	4.11 b	4.72 a	0.09	<0.001
efrB	4.20 b	3.92 b	3.97 b	4.60 a	0.08	<0.001
novA	3.46 bc	4.12 a	3.64 b	3.24 c	0.09	<0.001
vanRI	3.10 a	2.80 bc	2.59 c	2.91 ab	0.05	<0.001
TaeA	2.88	2.69	2.93	2.87	0.03	0.058
tet32	2.89 ab	2.77 ab	2.98 a	2.62 b	0.05	0.018
optrA	2.13 b	2.30 a	2.14 ab	2.24 ab	0.03	0.034
vatB	2.07	2.03	2.04	2.04	0.02	0.924
msbA	1.76 ab	1.54 b	1.90 a	1.73 ab	0.04	0.010
Total number[2]	3387.3	3394.3	3363.8	3357.5	10.37	0.460

[1]CON = Basal diet; ENM = Basal diet plus enramycin; SFP = Basal diet plus SFPs; LFP = Basal diet plus LFPs.
[2]Total number of antibiotic resistance genes.
a–c – mean values in the row without common letters are significantly different (P≤0.05).

Table 3. Effect of *Bacillus* species-fermented products on genera with a dominant assigned antibiotic resistance class and gene in the cecal digesta of broilers

		Relative abundance (%)				SEM	P value
		CON[1]	ENM	SFP	LFP		
Genus	Antibiotic resistance class						
Lachnoclostridium	peptide	26.8 a	25.0 ab	23.1 b	28.2 a	0.60	0.003
Blautia	glycopeptide	16.8 c	24.0 b	29.7 a	27.2 ab	1.37	<0.001
Clostridium	macrolide	29.0	27.6	25.3	28.2	0.62	0.167
Bacteroides	peptide	14.6	10.3	15.1	27.8 x	1.85	0.009
Genus	Antibiotic resistance gene						
Lachnoclostridium	*bcrA*	13.6 a	10.0 b	9.8 b	15.0 a	0.63	<0.001
Blautia	*efrA*	9.6 b	13.1 a	11.0 ab	9.2 b	0.47	0.002
Clostridium	*efrB*	17.2 b	18.6 ab	18.1 ab	21.6 a	0.60	0.037
Bacteroides	*rpoB2*	14.3 ab	10.2 c	14.8 a	10.4 bc	0.69	0.007

[1]CON = Basal diet; ENM = Basal diet plus enramycin; SFP = Basal diet plus SFPs; LFP = Basal diet plus LFPs.
a–c – mean values in the row without common letters are significantly different (P≤0.05, one-way analysis of variance followed by Tukey's honestly significant difference test).
x – LFP is significantly different to CON and ENM (P≤0.05, Kruskal–Wallis test followed by Dunn's pairwise test).

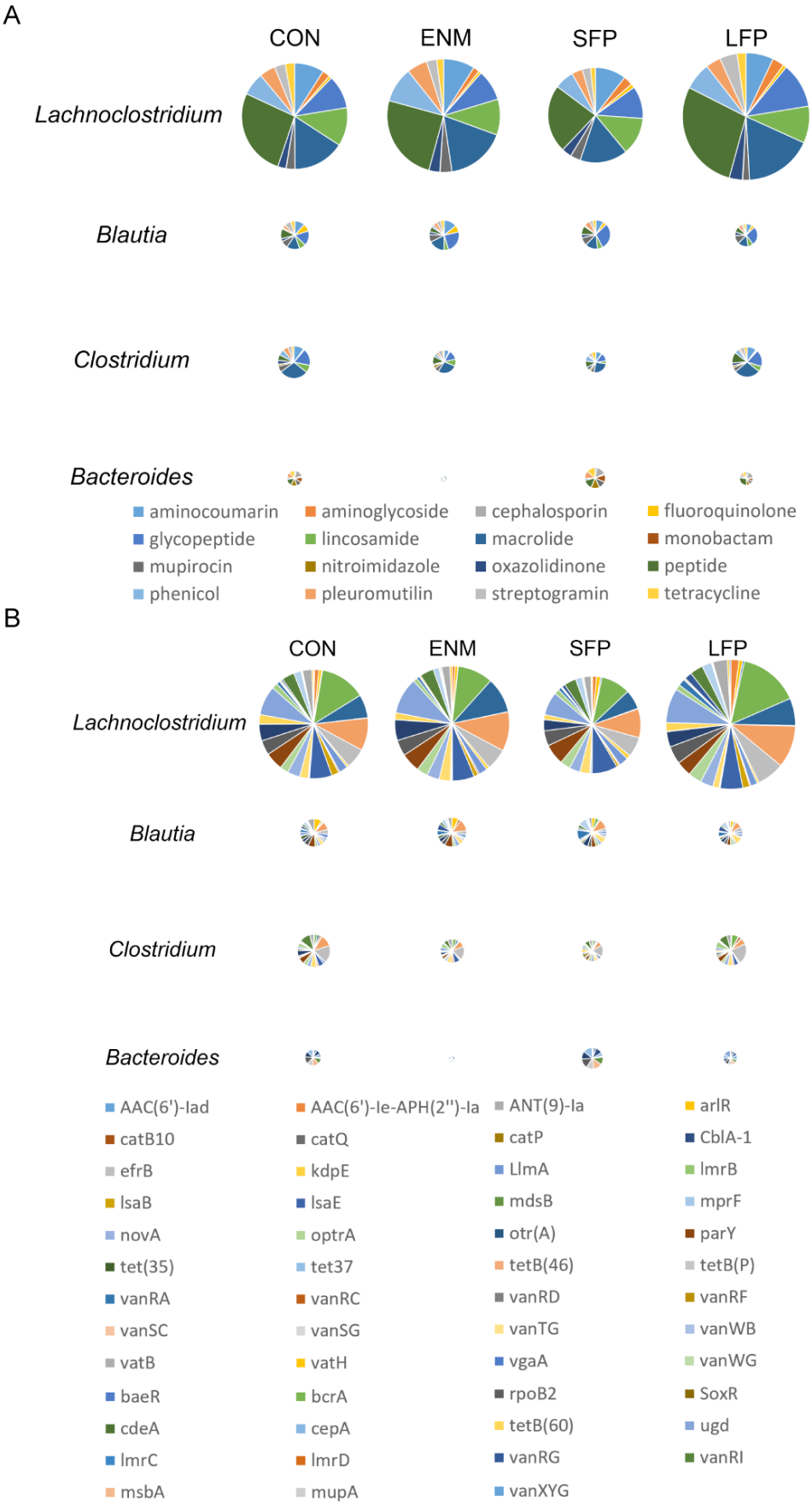

Figure 3. Pie charts displaying the distribution of the most abundant genera with (A) assigned antibiotic resistance genes from the main antibiotic resistance classes and (B) assigned antibiotic resistance genes. The size of each pie chart is proportional to the antibiotic resistance class or antibiotic resistance gene abundance within each group (n = 4)

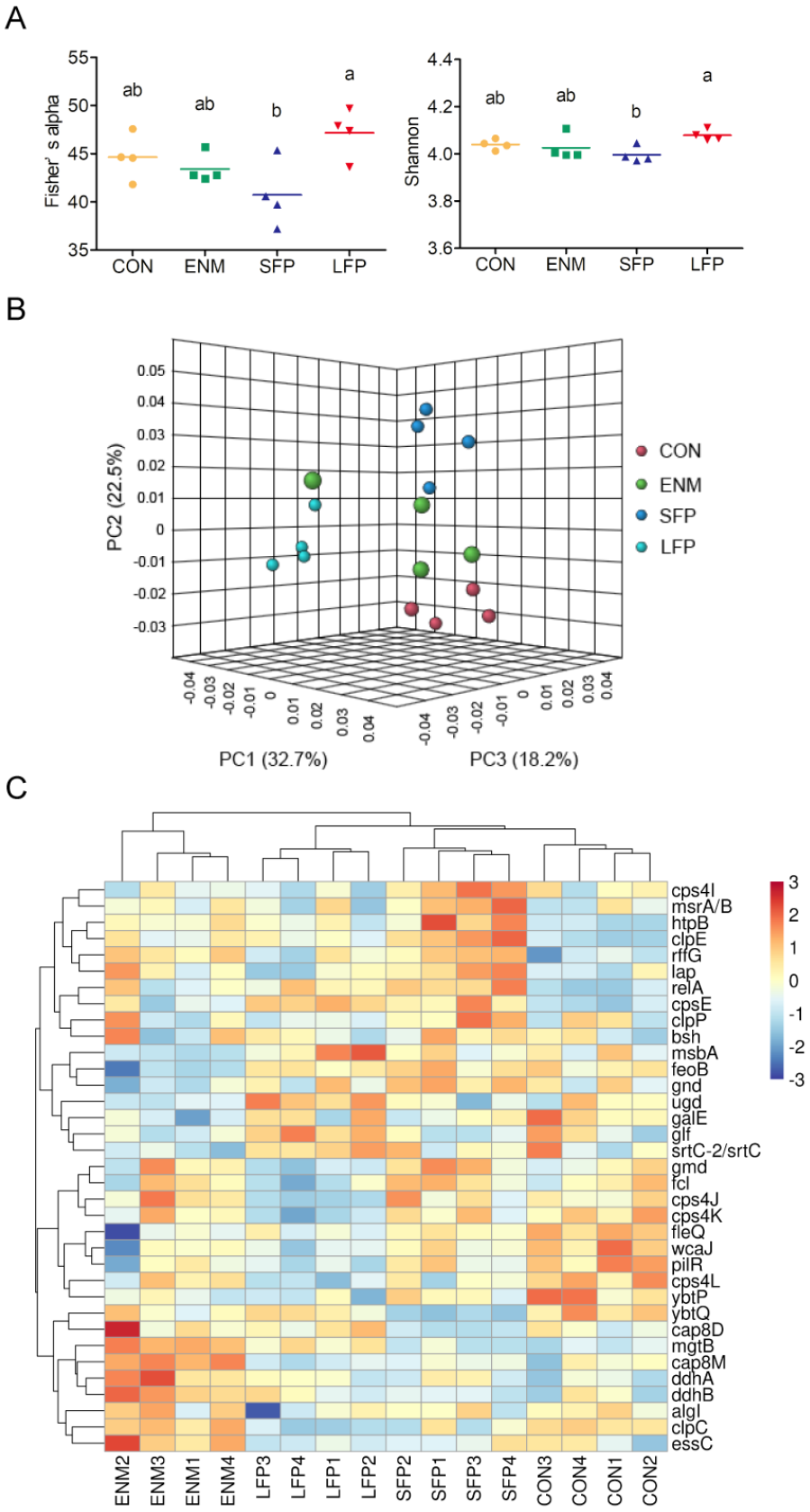

Figure 4. Comparison of the microbial virulence factor gene structure in the cecal digesta through advanced analysis. (A) Scatter plot of the alpha diversity of microbial virulence factor genes in CON (basal diet), ENM (basal diet plus enramycin), SFP (basal diet plus *B. subtilis*-fermented products), and LFP (basal diet plus *B. licheniformis*-fermented products) groups. Each bar represents the mean ± SEM (n = 4). Different superscripts indicate significant differences between the groups. (B) Principal coordinate analysis of the microbial virulence factor genes in the CON, ENM, SFP, and LFP groups (n = 4). (C) Heatmap of microbial virulence factor genes from cecal digesta. The abundance distribution of 35 dominant microbial virulence factor genes (Y-axis) across all samples (X-axis) is displayed (n = 4). The values are normalized using the Z-score

Table 4. Effect of *Bacillus* species-fermented products on virulence factor gene composition in the cecal digesta of broilers

	Relative abundance (%)				SEM	P value
	CON[1]	ENM	SFP	LFP		
clpC	10.9 y	11.0 x	10.5	10.2	0.09	0.017
algI	5.7	5.8	5.6	5.5	0.05	0.189
clpP	5.4	5.5	5.7	5.3	0.06	0.223
htpB	4.9 b	5.1 ab	5.3 a	5.0 b	0.04	0.003
galE	3.9	3.6	3.7	3.8	0.04	0.071
ugd	3.2 ab	3.0 b	3.1 b	3.5 a	0.06	0.004
glf	3.2 ab	3.0 b	3.0 b	3.4 a	0.05	0.023
rffG	2.9 b	3.3 a	3.3 a	3.0 ab	0.05	0.003
msbA	2.4 ab	2.3 b	2.4 ab	2.5 a	0.03	0.027
cps4I	2.4 ab	2.3 b	2.7 a	2.3 b	0.05	0.005
gmd	2.3 ab	2.3 ab	2.5 a	2.0 b	0.06	0.042
relA	1.9 b	2.1 ab	2.3 a	2.2 a	0.05	0.003
fcl	2.0	2.0	2.1	1.8	0.05	0.070
fleQ	2.3 a	1.7 b	2.0 ab	1.9 b	0.06	0.005
mgtB	1.8 c	2.3 a	1.7 c	2.0 b	0.06	<0.001
Total number[2]	344.3	336.3	340.0	350.8	1.62	0.787

[1]CON = Basal diet; ENM = Basal diet plus enramycin; SFP = Basal diet plus SFPs; LFP = Basal diet plus LFPs.
[2]Total number of virulence factor genes
a–c – mean values in the row without common letters are significantly different (P≤0.05, one-way analysis of variance followed by Tukey's honestly significant difference test).
x – ENM is significantly different to SFP and LFP; [y]CON is significantly different to LFP (P≤0.05, Kruskal–Wallis test followed by Dunn's pairwise test).

Table 5. Effect of *Bacillus* species-fermented products on genera with a dominant assigned virulence factor gene in the cecal digesta of broilers

		Relative abundance (%)					
		CON[1]	ENM	SFP	LFP	SEM	P value
Genus	Virulence factor gene						
Bacteroides	*clpC*	13.9 a	12.8 ab	13.0 ab	12.6 b	0.18	0.041
Faecalibacterium	*clpC*	18.6 a	18.4 a	16.9 b	17.7 ab	0.21	0.002
Lachnoclostridium	*clpC*	20.2	17.5	18.9	20.2	0.45	0.088
Lactobacillus	*clpE*	11.6	12.2	12.6	12.9	0.19	0.065

[1]CON = Basal diet; ENM = Basal diet plus enramycin; SFP = Basal diet plus SFPs; LFP = Basal diet plus LFPs.
a–b – mean values in the row without common letters are significantly different (P≤0.05).

Effect of SFPs and LFPs on microbial VFG composition

The richness and evenness of microbial VFGs were higher in the cecal digesta of the LFP group than in the SFP group (Figure 4 A). PCoA exhibited a clear separation of microbial VFG composition among the groups (Figure 4 B). Figure 4 C presents a heatmap illustrating the 35 most abundant VFGs in the cecal digesta of broilers. Specific VFGs, including *fleQ*, *wcaJ*, and *pilR*, were identified only in the CON group. Some VFGs, including *mgtB*, *cap8M*, and *ddhB*, were specifically identified in the ENM group. Specific VFGs, including *msrA/B*, *htpB*, and *clpE*, were identified only in the SFP group. Specific VFGs, including *ugd*, *glf*, and *srtC-2/srtC*, were identified only in the LFP group. Some VFGs, including *relA* and *cpsE*, specifically presented between the LFP and SFP groups. Similar VFGs, including *feoB* and *gnd*, were identified in the LFP, SFP, and CON groups. The effects of SFPs and LFPs on microbial VFGs in the cecal digesta of broilers are summarized in Table 4. The abundance of the caseinolytic protease C (*clpC*) gene was higher in the ENM group than in the LFP and SFP groups. *clpC* abundance was higher in the CON group than in the LFP group. *ugd* and *glf* abundance was lower in the ENM and SFP groups than in the LFP group. *htpB* abundance was higher in the SFP group than in the CON and LFP groups, but *rffG* abundance was lower in the CON group than in the ENM and SFP groups. *cps4I* and *gmd* abundance were higher in the SFP group than in the LFP group, but *msbA* abundance was lower in the ENM group than in the LFP group. *relA* abundance was higher in the SFP and LFP groups than in the CON group, but *fleQ* abundance was lower in the ENM and LFP groups than in the CON group. *mgtB* abundance was highest in the ENM group. No significant between-group differences in the total number of VFGs were identified (Table 4).

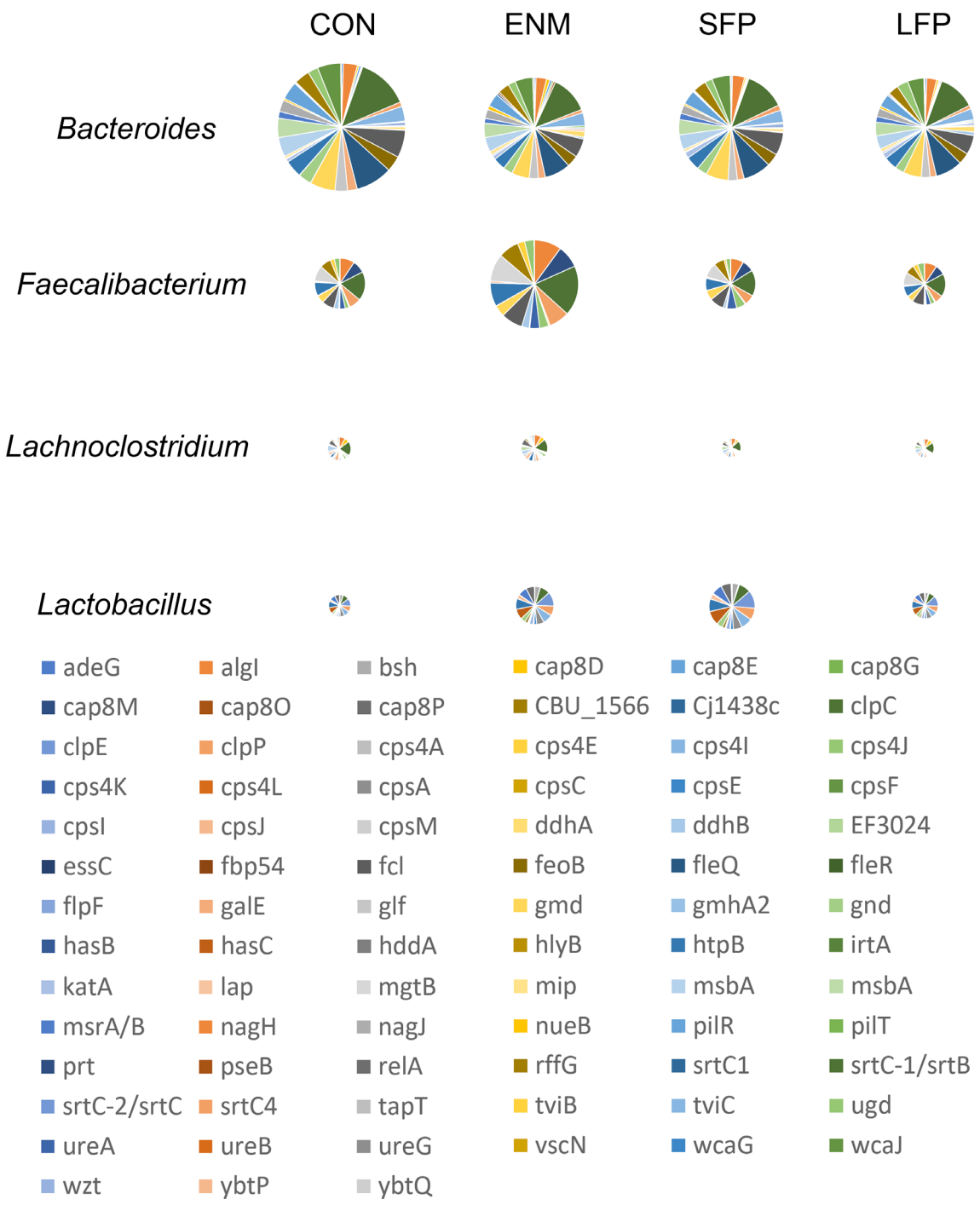

Figure 5. Pie charts displaying the distribution of the most abundant genera with assigned microbial virulence factor genes. The size of each pie chart is proportional to the abundance of microbial virulence factor genes within each group (n = 4)

Effect of SFPs and LFPs on the taxonomic assignment of microbial VFGs

Figure 5 presents the four most abundant genera with VFGs in the cecal digesta of broilers. In *Bacteroides*, *Faecalibacterium*, and *Lachnoclostridium* species, *clpC* was the most predominant VFG. *fleQ*, *mgtB*, and *algI* were the second most predominant VFGs in *Bacteroides*, *Faecalibacterium*, and *Lachnoclostridium* species, respectively. In *Lactobacillus* species, *clpE* and *htpB* were the most and second most predominant VFGs, respectively. The effects of SFPs and LFPs on the dominant genera with the dominant assigned VFGs in the cecal digesta of broilers are summarized in Table 5. *clpC* abundance in *Bacteroides* species was higher in the CON group than in the LFP group. *clpC* abundance in *Faecalibacterium* species was lower in the SFP group than in the CON and ENM groups.

Discussion

Prophylactic use of antibiotics in broiler feed for disease prevention and body weight gain accelerates the emergence and spread of antimicrobial resistance (Sreejith et al., 2020). Antibiotic-resistant bacteria can transfer their ARGs to enteric bacteria in broiler guts (Zalewska et al., 2021). Therefore, the healthy gut microbial composition of broilers is eventually replaced by antibiotic-resistant bacteria after exposure to antibiotic growth promoters (Sreejith et al., 2020). *Bacillus*-based probiotics and antibiotic growth promoters have been reported to differentially modulate the gut microbial composition of broilers (Qiu et al., 2021). Our previous study demonstrated that LFP and enramycin differentially modulate the antibiotic resistome in the cecal digesta of broilers, even though both lead to similar growth performance (Chen and Yu, 2022 b). Different from our previous study (Chen and Yu, 2022 b), in the present study, we observed the differential regulation of antibiotic resistomes in the cecal digesta of broilers treated with LFPs. Our previous study demonstrated that the richness of total antibiotic resistance classes was reduced in the cecal digesta of broilers treated with LFPs (Chen and Yu, 2022 b), whereas this richness was not altered by LFP supplementation in the present study. Our observations of the abundance of individual antibiotic resistance classes and individual ARGs were different to those in our previous study (Chen and Yu, 2022 b). Similar to our previous study (Chen and Yu, 2020), the growth-promoting effect of LFP in broilers was also observed in the current study (these data are presented by Chen and Yu, 2023), although the concentration of *B. licheniformis* spore was different. However, the cecal microbiota composition of broilers in response to LFP supplementation was slightly different in these two studies (Chen and Yu, 2020, 2023). The different dominant genera with assigned antibiotic resistance classes and ARGs may explain the inconsistent effects on the cecal antibiotic resistome in these studies. Bacterial community shifts are highly associated with antibiotic-resistance alterations in broilers (Xiong et al., 2018). Our data indicated that SFP and LFP differentially regulate the cecal bacterial community in broilers and that SFP and LFP supplementation had distinct regulation patterns in individual antibiotic resistance classes and individual ARGs. For example, LFP supplementation increased the abundance of peptide resistance genes compared with SFP supplementation, and the opposite was true for the abundance of aminocoumarin resistance genes. The antibacterial mechanism of *B. subtilis* or *B. subtilis*-derived metabolites mainly targets the cell membrane of pathogens, whereas *B. licheniformis* or *B. licheniformis*-derived metabolites mainly target the pathogens' cell wall and quorum sensing (Tran et al., 2022). Distinct antimicrobial mechanisms may differentially influence the antibiotic resistome. Taken together, these findings demonstrate that SFP and LFP supplementation exhibit a differential antibiotic resistome in the cecal digesta of broilers.

The caseinolytic protease C (*clpC*) gene encodes an ATP-dependent serine protease that has proteolytic and chaperone functions (Wawrzynow et al., 1999; Choules et al., 2019). Deletion of *clpC* or alteration of the caseinolytic protease function can affect the virulence and infectivity of pathogens (Cassenego et al., 2016). *clpC* regulates several key steps of the developmental processes of bacteria, such as competence (Turgay et al., 1998), sporulation (Pan et al., 2001), and stress response (Krüger et al., 2000). In the present study, SFP and LFP supplementation reduced the abundance of *clpC* in the cecal digesta of broilers. On the basis of the previous study (Chen and Yu, 2023), *clpC* abundance in the cecal digesta was negatively correlated with body weight ($r = -0.58$), average daily gain ($r = -0.60$), and average daily feed intake ($r = -0.31$; data were analyzed using Pearson correlation coefficient) of broilers. SFP and LFP supplementation inhibited *clpC* abundance in *Bacteroides* species and *Faecalibacterium* species, respectively. We previously demonstrated that LFPs reduced the relative abundance of the genus *Bacteroides* (the most predominant species) in the cecal digesta of broilers (Chen and Yu, 2023). Therefore, we speculate that the total amount of bacteria carrying *clpC* in the LFP group is much lower than those in the other groups. The diminished *clpC* abundance may accompany a reduced risk of toxin-induced damage in the intestinal epithelial cells of broilers, thereby lowering gut inflammation and promoting nutrient utilization for growth. By contrast, SFP supplementation does not change the relative abundance of the genus *Faecalibacterium* (second most predominant species) in the cecal digesta of broilers but does reduce *clpC* abundance in *Faecalibacterium* species. Hence, the risk of toxin-induced damage in intestinal epithelial cells in the SFP group may be higher than that in the LFP group. This may also explain why broiler growth performance in the LFP group was better than that in the SFP group in our previous study (Chen and Yu, 2023). Taken together, our results indicate that SFPs and LFPs differentially regulate microbial VFG composition and distribution in the cecal digesta of broilers. Both SFP and LFP supplementation inhibited *clpC* abundance in the cecal digesta of broilers, thus affecting their growth performance.

Supplementation with plant extracts can modulate broiler gut microbiota, which leads to the alteration of the ARG-harboring bacterial host composition (Huang et al., 2018; Koorakula et al., 2022). Changes in microbial community structure are associated with microbial VFG composition (Noman et al., 2022). In addition, ARGs are positively associated with certain VFGs in both commensal and pathogenic bacteria in the gut of broilers (Szmolka et al., 2012; Lee et al., 2021). Dietary supplementation of *Bacillus* species may regulate the broiler gut microbiome through the competitive exclusion of pathogens, neutralization of toxins, production of antimicrobial substances or extracellular enzymes, and immunomodulation in the gut mucosal immune system (Ramlucken et al., 2020). *B. subtilis* and *B. li-*

cheniformis produce different extracellular enzymes, metabolites, and antimicrobial substances (Sumi et al., 2015; Elshaghabee et al., 2017; Tran et al., 2022). For example, *B. licheniformis* exhibits higher lipase activity, whereas *B. subtilis* displays higher nuclease and phosphatase activity (Elshaghabee et al., 2017). These differences in extracellular enzymes may lead to differences in the growth of beneficial microbes in the gut of broilers. Furthermore, metabolites and antimicrobial substances generated by *B. subtilis* and *B. licheniformis* exhibit different antimicrobial mechanisms, thereby suppressing different pathogens in the gut of broilers. *B. subtilis* can produce substances with antimicrobial activity against Gram-positive and Gram-negative bacteria, such as subtilin and ericin (Stoica et al., 2019). By contrast, *B. licheniformis* mainly secretes antimicrobial substances against Gram-positive bacteria, such as lichenicidin (Stoica et al., 2019). We previously reported that SFPs and LFPs differentially modulated cecal microbial composition in broilers (Chen and Yu, 2023). Thus, the differences in characteristics between the two *Bacillus* species induce a distinct cecal microbial composition, affecting ARGs and VFG-harboring bacterial host composition. Whether these changes directly affect broiler health and growth warrants further research. Pedroso et al. (2013) concluded that administering alternatives to antibiotics can alter litter microbial community and diminish the abundance of pathogenic bacteria in broilers but may not immediately reduce the prevalence of antibiotic resistance. Thus, the full benefits of SFPs and LFPs in poultry production may require many cycles of production to lower the prevalence of ARG and VFG in farm environments.

The inconsistent results of resistome between the current study and our previous study are observed (Chen and Yu, 2022 b), specifically the effects of LFPs on resistome in the cecal digesta. The possible explanation for the difference is dosage of LFPs in the diet of broilers. The concertation of LFPs is 10^8 CFU of *B. licheniformis* spores/kg and 9×10^9 CFU of *B. licheniformis* spores/kg in the current study and our previous study, respectively. Different concentration of *B. licheniformis* spores in the diet may bring differential changes in gut microbiota composition that lead to changes in the abundance of bacterial hosts carrying specific ARGs. Results from cecal microbiota also support the speculation that differential gut microbiota composition is observed between these two studies (Chen and Yu, 2022 b, 2023).

Conclusions

We demonstrated for the first time that a distinct microbial ARG and VFG composition was observed in the cecal digesta of broilers treated with SFPs compared with those treated with LFPs. Our results provide insights into how SFPs and LFPs differentially affect ARG- and VFG-harboring bacterial host composition. Taken together, these findings further deepen our understanding of *Bacillus* species-fermented products in broilers and may guide the development of safe and effective alternatives to antibiotic growth promoters.

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