






Effects of therapeutic levels of dietary antibiotics on the cecal microbiome composition of broiler chickens

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ABSTRACT Dietary antibiotics, including antibiotic growth promoters (**AGPs**), have been commonly used to improve health and growth of poultry. The present study investigated the effects of therapeutic doses of dietary antibiotics, including bacitracin methylene disalicylate (**BMD**), penicillin G potassium (**PP**) and an ionophore (salinomycin, **SA**), on the cecal microbiome of chickens. BMD and SA treatments were given as dietary supplements from d 1 to 35 of age. The SAPP (salinomycin+ penicillin G potassium) group was given SA as a dietary supplement from d 1 to 35 of age and PP was added to drinking water from d 19 to 24 of age to simulate common practices for control of necrotic enteritis in broilers. The cecal contents were collected from all treatment groups on d 10, 24, and 35 of age and DNA was extracted for metagenomic analysis of the cecal microbiome. The results revealed that dietary or water supplementation of therapeutic levels of antibiotics and ionophores to chickens significantly altered the cecal microbial homeostasis during different stages of

the chicken life. The alpha diversity analysis showed that BMD, SA, and SAPP treatments decreased diversity and evenness of the cecal microbiome of treated chickens on d 10 of age. Species richness was also reduced on d 35 following treatment with BMD. Beta diversity analyses revealed that SAPP and BMD induced significant changes in the relative abundance of Gram-positive and -negative bacteria on d 10, while no significant differences were observed on d 24. On d 35, the non-treated control group had higher relative abundance of unclassified Gram-positive and -negative bacteria compared to SA, SAPP, and BMD treatment groups. Overall, despite their beneficial role in controlling necrotic enteritis outbreaks, the findings of this study highlight the potential negative effects of dietary supplementation of therapeutic levels of antibiotics on the gut microbiome and suggest that adjusting gut bacteria may be required to restore microbial richness and diversity of the gut microbiome following treatment with these antibiotics.

Key words: microbiome, antibiotics, ionophore, growth promoter, chicken

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INTRODUCTION

Antimicrobial growth promoters (**AGPs**) have been used widely as feed additives to improve livestock health and productivity (Dibner and Richards, 2005). In addition to their health benefits, inclusion of subtherapeutic levels of antibiotics in poultry feed has been shown to

enhance feed efficiency and growth performance and to reduce the levels of enteric bacterial pathogens, including *Clostridium perfringens* (Gadbois et al., 2008; Neumann and Suen 2015). However, due to public health concerns about the impact of antibiotic residues on human health and the potential emergence of antibiotic-resistant bacteria, many countries have taken steps to mitigate these risks by phasing out the use of certain antibiotics in poultry feed (United States Food and Drug Administration 2012; Agunos et al., 2019). For example, measures have been set in Canada to eliminate the preventive use of antimicrobials in poultry feed, including Category I, II, III antibiotics, while Category

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IV antibiotics such as ionophores that are not being used for humans will not continue to be used in the raised without antibiotic (**RWA**) programs. The removal of antibiotics from poultry feed has been thought to result in re-emergence of some well-controlled diseases such as necrotic enteritis (**NE**), consequently leading to the use of antibiotics at therapeutic doses for treatment of these diseases.

Classical AGPs including bacitracin methylene disalicylate (**BMD**) and ionophores such as salinomycin (**SA**) are commonly used in poultry production to prevent or control infectious diseases caused by Gram-positive bacteria, particularly *C. perfringens*-induced NE (Johansen et al., 2007). Administration of penicillin G potassium (Pot-Pen, **PP**) has also been shown to reduce the incidence of NE in broiler chickens, in addition to its non-specific growth-promoting effect (Gadbois et al., 2008). When subtherapeutic doses are supplemented in diet, these antibiotics exert their effects either directly through inhibition of bacterial cell wall synthesis or disintegration of the cell membrane of Gram-positive bacteria and/or indirectly through inducing significant alterations in the gut mucosal immunity and microbiome composition (Broom, 2017).

In recent years, 16S rRNA surveys, driven by next generation sequencing technology tools, have enabled researchers to distinguish the changes in the gut microbiota profiles in response to dietary changes. In this context, recent studies have shown that dietary supplementation of AGPs results in substantial reduction in the abundance of *C. perfringens* and other Gram-positive bacteria such as Lactobacilli, Bifidobacteria, and *Streptococcus*, which make up the majority of beneficial bacteria in the gastrointestinal tract (Broom, 2017), and proliferation of Gram-negative bacteria, including *Salmonella* and *Campylobacter*, perhaps due to the lack of competition for available nutrients (Kumar et al., 2019).

While the effects of subtherapeutic doses of antibiotics on the chicken gut microbiome composition and diversity have been extensively studied, relatively little is known about the impact of therapeutic levels of antibiotics on the chicken gut microbiome dynamics. A better understanding of the possible antibiotic-associated dynamic shifts of the gut microbiome will not only aid in the prudent use of antibiotics in poultry production, but will also help in developing potential therapeutics, such as probiotics, to re-shape an impaired gut microbiome following their use. Therefore, this study was undertaken to investigate the effects of different antibiotics, including bacitracin methylene disalicylate, salinomycin, and penicillin G potassium, at therapeutic dosages on the cecal microbiome composition using next generation sequencing.

MATERIALS AND METHODS

Experimental Design

Eighty one-day-old mixed broiler chicks (Ross 708), obtained from a commercial hatchery in Ontario,

Table 1. Starter diet formulation for broilers (d 1–10) fed bacitracin methylene disalicylate or salinomycin or no antibiotics.

Ingredients, %	Starter			
	Control	BMD	Salinomycin	
Salinomycin + Pot Pen ¹				
Corn	41.98	41.83	41.93	41.93
Soybean meal 46% CP	32.87	32.87	32.87	32.87
Wheat	10	10	10	10
Corn gluten meal	5	5	5	5
Meat & bone meal	0.4	0.4	0.4	0.4
Choline chloride, 60%	0.25	0.25	0.25	0.25
Limestone	1.55	1.55	1.55	1.55
Iodized salt	0.15	0.15	0.15	0.15
Mono-dicalcium phosphorus	1.69	1.69	1.69	1.69
Sodium bicarbonate	0.36	0.36	0.36	0.36
L-Lysine-HCl	0.35	0.35	0.35	0.35
DL-Methionine	0.31	0.31	0.31	0.31
L-Threonine	0.13	0.13	0.13	0.13
Vitamin-mineral premix ²	1	1	1	1
Soybean oil	3.96	3.96	3.96	3.96
BMD ³ , %	0	0.15	0	0
Salinomycin ⁴ , %	0	0	0.05	0.05
Total	100	100	100	100
Calculated provisions				
AME _n (kcal/kg)	3,000	3,000	3,000	3,000
Crude protein, %	24.00	24.00	24.00	24.00
Crude fat, %	6.72	6.72	6.72	6.72
Calcium, %	0.96	0.96	0.96	0.96
Available phosphorus, %	0.48	0.48	0.48	0.48
dLysine, %	1.29	1.29	1.29	1.29
dThreonine, %	0.87	0.87	0.87	0.87
dMethionine, %	0.65	0.65	0.65	0.65
dMethionine + Cysteine, %	0.96	0.96	0.96	0.96
Na, g/kg	0.16	0.16	0.16	0.16

¹20 grams of penicillin G potassium (Pot-Pen)/100 liter of drinking water (SA+PP) from day 19 to 24 of age, Bio Agri Mix.

²Vitamin/mineral premix contains the following per kg of diet: trans-retinol, 2.64 mg; cholecalciferol, 83 µg; dl-α-tocopherol, 36 mg; cyanocobalamin, 12.0 mg; menadione, 3.3 mg; niacin, 50.0 mg; choline, 1,200.0 mg; folic acid, 1.0 mg; biotin, 0.22 mg; pyridoxine, 3.3 mg; thiamine, 4.0 mg; calcium pantothenic acid, 15.0 mg; riboflavin, 8.0 mg; manganese, 70.0 mg; zinc, 70.0 mg; iron, 60.0 mg; iodine, 1.0 mg; copper, 10 mg; and selenium, 0.3 mg.

³0.15% Medicated Bacitracin Methylene Disalicylate Premix (BMD), BMD 110G, Zoetis Inc.

⁴0.05% Medicated Coxistac 12% Granular, Phibro Animal Health Corporation.

Canada, were randomly assigned to 4 groups (n = 20/group) and housed in separate floor pens (200 × 215 cm) with fresh litter in the same room at Arkell poultry research station, University of Guelph. Chickens in group 1 were fed a diet containing 0.15% BMD at (150 g/ton) (Bean-Hodgins and Kiarie, 2021) during each phase of growth: starter (d 1 to 10 of age; Table 1), grower (d 11 to 24 of age; Table 2), and finisher (d 25 to 35 of age; Table 3). Chickens in group 2 were fed a diet containing 0.05% SA at (50 g/ton) (Bean-Hodgins and Kiarie, 2021) during the starter, grower and finisher phases. Chickens in group 3 were given a diet containing 0.05% salinomycin from d 1 to 35 of age and had access to medicated water containing 20 g of penicillin g potassium (Pot-Pen)/100 liter of drinking water (SA + PP) from d 19 to 24 of age. Chickens in group 4 served as a negative control group and did not receive any medicated feed or water throughout the entire experiment. The room temperature was maintained at 32°C during

Table 2. Grower diet formulation for broilers (d 11–24) fed bacitracin methylene disalicylate or salinomycin or no antibiotics.

Ingredients, %	Grower			
	Control	BMD	Salinomycin	Salinomycin + Pot Pen ¹
Corn	47.07	46.92	47.02	47.02
Soybean meal 46% CP	28.36	28.36	28.36	28.36
Wheat	10	10	10	10
Corn gluten meal	4.15	4.15	4.15	4.15
Choline chloride, 60%	0.22	0.22	0.22	0.22
Limestone	1.49	1.49	1.49	1.49
Iodized salt	0.16	0.16	0.16	0.16
Mono-dicalcium phosphorus	1.61	1.61	1.61	1.61
Sodium bicarbonate	0.35	0.35	0.35	0.35
L-Lysine-HCl	0.32	0.32	0.32	0.32
DL-Methionine	0.28	0.28	0.28	0.28
L-Threonine	0.11	0.11	0.11	0.11
Vitamin-mineral premix ²	1	1	1	1
Soybean Oil	4.88	4.88	4.88	4.88
Pork Meal	0	0	0	0
BMD ³ , %	0	0.15	0	0
Salinomycin ⁴ , %	0	0	0.05	0.05
Total	100	100	100	100
Calculated provisions				
AME _n (kcal/kg)	3,100	3,100	3,100	3,100
Crude protein, %	21.50	21.50	21.50	21.50
Crude fat, %	7.65	7.65	7.65	7.65
Calcium, %	0.87	0.87	0.87	0.87
Available phosphorous, %	0.44	0.44	0.44	0.44
dLysine, %	1.15	1.15	1.15	1.15
dThreonine, %	0.77	0.77	0.77	0.77
dMethionine, %	0.58	0.58	0.58	0.58
dMethionine + Cysteine, %	0.87	0.87	0.87	0.87
Available phosphorous, %	0.44	0.44	0.44	0.44
Na, g/kg	0.16	0.16	0.16	0.16

¹20 grams of penicillin G potassium (Pot-Pen)/100 liter of drinking water (SA+PP) from day 19 to 24 of age, Bio Agri Mix.

²Vitamin/mineral premix contains the following per kg of diet: trans-retinol, 2.64 mg; cholecalciferol, 83 µg; dl- α -tocopherol, 36 mg; cyanocobalamin, 12.0 mg; menadione, 3.3 mg; niacin, 50.0 mg; choline, 1,200.0 mg; folic acid, 1.0 mg; biotin, 0.22 mg; pyridoxine, 3.3 mg; thiamine, 4.0 mg; calcium pantothenic acid, 15.0 mg; riboflavin, 8.0 mg; manganese, 70.0 mg; zinc, 70.0 mg; iron, 60.0 mg; iodine, 1.0 mg; copper, 10 mg; and selenium, 0.3 mg.

³0.15% Medicated Bacitracin Methylene Disalicylate Premix (BMD), BMD 110G, Zoetis Inc.

⁴0.05% Medicated Coxistac12% Granular, Phibro Animal Health Corporation.

the first week of hatch and gradually decreased to reach 27°C by d 17. Birds were exposed to fluorescent lighting in a 23 h of light (20+ lux) for the first 4 days and then a 16 light: 8 dark (10–15 lux) light cycle. Birds in all groups had free access to the food and water during the experiment. The experiment complied with the guidelines of the Canadian Council on Animal Care (CCAC) and was approved by the Animal Care Committee at the University of Guelph.

DNA Extraction and Sequencing

On d 10, 24, and 35 of age, five birds randomly collected from each treatment group and euthanized by CO₂ inhalation. Cecal contents were collected from both ceca, snap-frozen on dry ice and then stored at –80°C until further use (Zhou et al., 2007). DNA was extracted from the cecal contents using QIAamp DNA Stool mini

kit (Cat. No. / ID:51604) as per manufacturer's instruction (Qiagen, Toronto, Canada). DNA quality and concentration were assessed with a NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE). The DNA samples with 260/280 ratio between 1.8 and 2 were considered pure and were used for further processing. DNA concentration was measured and adjusted to 10 ng/µL in a volume of 10 µL nuclease-free water. The V4 hypervariable region of the 16S rRNA gene was amplified and sequenced with an Illumina MiSeq sequencer at the Genome Quebec Innovation Center. The pair primer set used in the study was 564F YTG GGYDTAAAGNG and 785R TACNVGGG-TATCTAATCC. A total of 1,359,182 sequences were produced with an average number of 22,653 sequences per sample.

Sequence Processing and Bioinformatics Analysis

Processing of sequence data was performed using Mothur (v.1.44.) (Schloss, 2009) software, following the MiSeq SOP (Kozich et al., 2013). Briefly, make.contigs command was applied to assemble the read pairs into contigs. Non-assembled and redundant contigs were removed by screen.seqs and unique.seqs commands, respectively. The sequences that did not align to V4 region of the 16S rRNA gene and had ambiguous bases less than 254 bp or were not classified as bacteria or contained homopolymers of >9 bp removed. No samples were removed due to low reads. SILVA bacteria reference database 138 was used to align the sequences. The redundant aligned sequences created by trimming the ends were removed by unique.seqs command. The chimera.vsearch command was used to remove the chimeric sequences. The dist.seqs (cutoff = 0.03) and cluster commands were used to cluster the sequences into operation taxonomic units (OTUs), and the number of sequences per OTU was determined by using the make.shared command. Taxonomy was assigned against SILVA database (v.138.). Alpha and beta microbial community diversity analysis were performed using standard Mothur pipelines. Bacterial community dissimilarity in the treatments was measured by Nonmetric multidimensional scaling (NMDS) using Bray Curtis dissimilarity index.

Permutational multivariate analysis of variance (PERMANOVA) ('Adonis' function, vegan package, R [v.3.5.1.]; 1,000 permutations) was used to analyze statistical comparison of spatial separation on the nMDS. The significant differences between the taxa associated with each treatment were analyzed with the package DESeq2 (Love et al., 2014) in R software.

RESULTS

Alpha Diversity of the Cecal Microbiome

Alpha diversity Chao1 (richness measures), and Shannon and Simpson diversity (evenness measures) were

Table 3. Finisher diet formulation for broilers (d 25–35) fed bacitracin methylene disalicylate or salinomycin or no antibiotics.

Ingredients, %	Finisher			
	Control (%)	BMD (%)	Salinomycin (%)	Salinomycin (%) + Pot Pen ¹
Corn, %	55.40	55.25	55.35	55.35
Soybean meal 46% CP	21.69	21.69	21.69	21.69
Wheat	7.76	7.76	7.76	7.76
Corn gluten meal	3.4	3.4	3.4	3.4
Choline chloride, 60%	0.20	0.20	0.20	0.20
Limestone	0.89	0.89	0.89	0.89
Iodized salt	0.25	0.25	0.25	0.25
Mono-dicalcium phosphorus	0.84	0.84	0.84	0.84
Sodium bicarbonate	0.16	0.16	0.16	0.16
L-Lysine-HCl	0.31	0.31	0.31	0.31
DL-Methionine	0.26	0.26	0.26	0.26
L-Threonine	0.10	0.10	0.10	0.10
Vitamin-mineral premix ²	1	1	1	1
Soybean oil	5	5	5	5
Pork meal-60%	2.74	2.74	2.74	2.74
BMD ³ , %	0	0.15	0	0
Salinomycin ⁴ , %	0	0	0.05	0.05
Total	100	100	100	100
Calculated provisions				
AME _n (kcal/kg)	3,200	3,200	3,200	3,200
Crude Protein, %	19.75	19.75	19.75	19.75
Crude Fat, %	8.18	8.18	8.18	
Calcium, %	0.79	0.79	0.79	0.79
dLysine, %	1.03	1.03	1.03	1.03
dThreonine, %	0.69	0.69	0.69	0.69
dMethionine (%)	0.55	0.55	0.55	0.55
dMethionine + Cysteine, %	0.80	0.80	0.80	0.80
Available phosphorous, %	0.39	0.39	0.39	0.39
Na, g/kg	0.16	0.16	0.16	0.16

¹20 grams of penicillin G potassium (Pot-Pen)/100 liter of drinking water (SA+PP) from d 19 to 24 of age, Bio Agri Mix.

²Vitamin/mineral premix contains the following per kg of diet: trans-retinol, 2.64 mg; cholecalciferol, 83 µg; dl- α -tocopherol, 36 mg; cyanocobalamin, 12.0 mg; menadione, 3.3 mg; niacin, 50.0 mg; choline, 1,200.0 mg; folic acid, 1.0 mg; biotin, 0.22 mg; pyridoxine, 3.3 mg; thiamine, 4.0 mg; calcium pantothenic acid, 15.0 mg; riboflavin, 8.0 mg; manganese, 70.0 mg; zinc, 70.0 mg; iron, 60.0 mg; iodine, 1.0 mg; copper, 10 mg; and selenium, 0.3 mg.

³0.15% Medicated Bacitracin Methylene Disalicylate Premix (BMD).

⁴0.05% Medicated Coxistac12% Granular, Phibro Animal Health Corporation.

carried out using Mothur software. On d 10 (Figure 1), alpha diversity indexes indicated that chickens in the SA, BMD, and SAPP treatment groups had significantly different cecal microbial evenness and diversity compared to the non-treated control group. The Shannon index showed that chickens in the control group had higher microbial evenness ($P < 0.05$, ANOVA) compared to those in SAPP group and marginal evenness compared to those in the SA treatment group (Figure 1). Chickens in the SAPP treatment group had lower evenness compared to those in the SA treatment group (Figure 1).

Based on inverse Simpson's, chickens in the control group had higher microbial diversity ($P < 0.05$, ANOVA) in comparison with those in the SAPP treatment group (Figure 1). Further, the Chao diversity index demonstrated that species richness of the cecal bacterial community was not affected by any of the antibiotic treatments (Figure 1).

On d 24, the Chao, Shannon, and Inverse Simpson indexes revealed that none of the antibiotic treatments significantly altered either richness or evenness of the cecal bacterial community (Figure 2).

On d 35 (Figure 3), the Chao and Shannon indexes indicated that richness and evenness of the cecal bacteria community in the control group was significantly

higher ($P < 0.05$, ANOVA) than BMD treatment group (Figure 3). The inverse Simpson index did not show any changes in the evenness of the cecal bacterial community by any of the antibiotic treatments (Figure 3).

There were not any species shared among the control, SA, and SAPP groups on d 10, 24, and 35 (Figures 4–6).

Beta Diversity of the Cecal Microbiome

Beta diversity of the cecal microbiome in chickens was measured in NMDS with Bray Curtis index on d 10, 24, and 35 (Figure 7). Analysis of PERMANOVA was performed to determine the difference in the cecal bacterial communities among treatment groups (Table 4). All pairwise comparisons were significant using ADONIS at 0.05 using Benjamini-Hochberg correction for multiple comparison.

On d 10, the cecal microbial community structure of the SA-treated chickens was significantly dissimilar from the control group (Table 4). SA-treated chickens were different in the cecal microbial structure compared to the SAPP group (Table 4).

On d 24, the cecal microbial community structure of the SA-treated chickens was different from that of the SAPP-treated and control groups (Table 4).

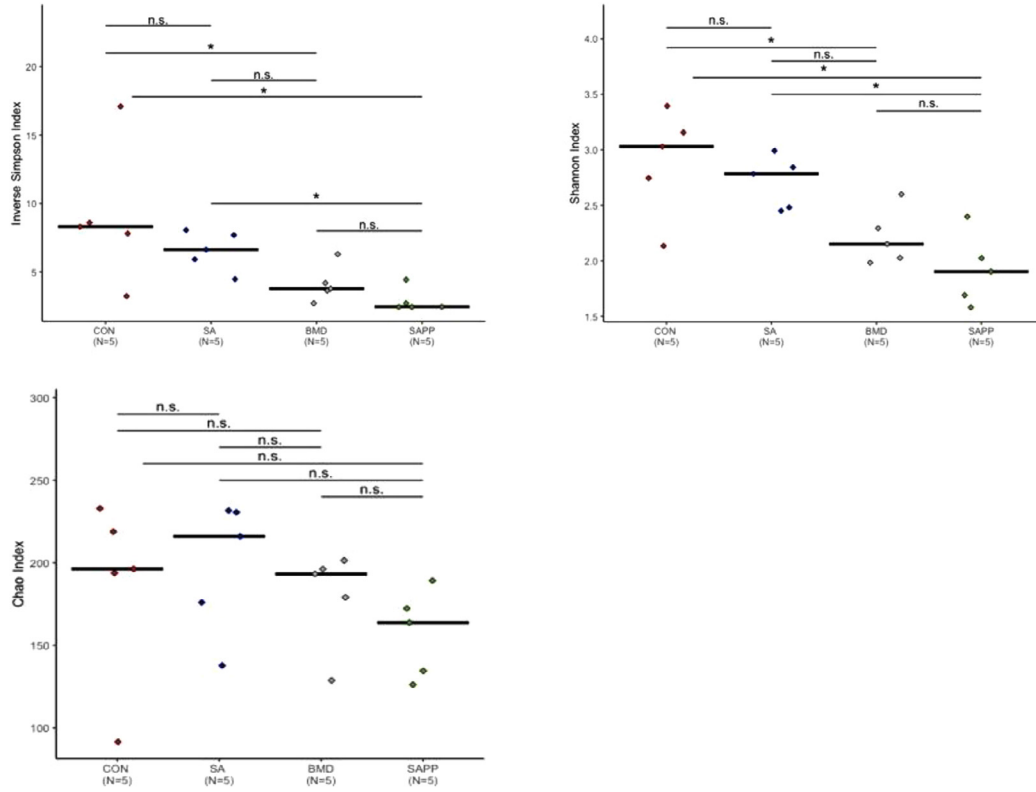


Figure 1. Alpha diversity analysis illustrating evenness, richness and diversity measures of cecal microbiome of chickens on d 10 of receiving a diet containing 0.15% bacitracin methylene disalicylate (BMD) at (150 g/ton) during the growing phase: starter (d 1 to 10 of age), grower (d 11 to 24 of age), and finisher (d 25 to 35 of age), or a diet containing 0.05% salinomycin (SA) at (50 g/ton) during the growing phase from d 1 to 35 of age, or a diet containing 0.05% salinomycin from d 1 to 35 of age and had access to medicated water containing 20 grams of penicillin G potassium (Pot-Pen)/100 liter of drinking water (SA + PP) from d 19 to 24 of age, or did not receive any medicated feed or water throughout the entire experiment (a negative control group). * indicates a significant difference at the 0.05 level between CON, SA, SAPP, and BMC that were compared. n.s. indicate a no significant difference at less than 0.05 between CON, SA, SAPP, and BMD that were compared.

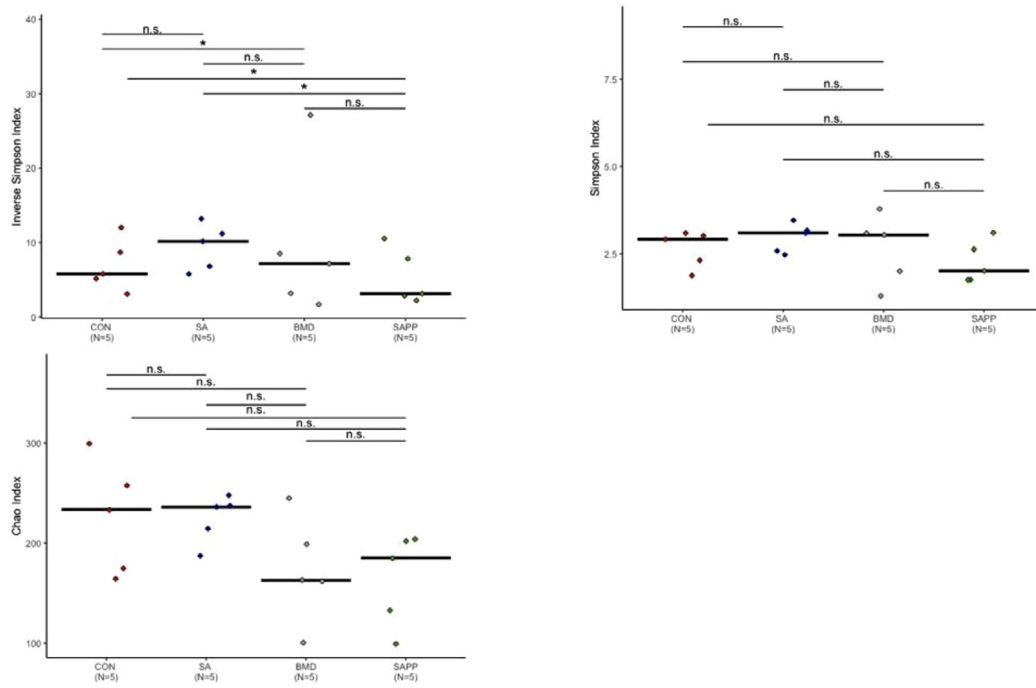


Figure 2. Alpha diversity analysis illustrating evenness, richness and diversity measures of cecal microbiome of chickens on d 24 of receiving a diet containing 0.15% bacitracin methylene disalicylate (BMD) at (150 g/ton) during the growing phase: starter (d 1 to 10 of age), grower (d 11 to 24 of age), and finisher (d 25 to 35 of age), or a diet containing 0.05% salinomycin (SA) at (50 g/ton) during the growing phase from d 1 to 35 of age, or a diet containing 0.05% salinomycin from d 1 to 35 of age and had access to medicated water containing 20 grams of penicillin G potassium (Pot-Pen)/100 liter of drinking water (SA + PP) from d 19 to 24 of age, or did not receive any medicated feed or water throughout the entire experiment (a negative control group). * indicates a significant difference at the 0.05 level between CON, SA, SAPP, and BMC that were compared. n.s. indicate a no significant difference at less than 0.05 between CON, SA, SAPP, and BMD that were compared.

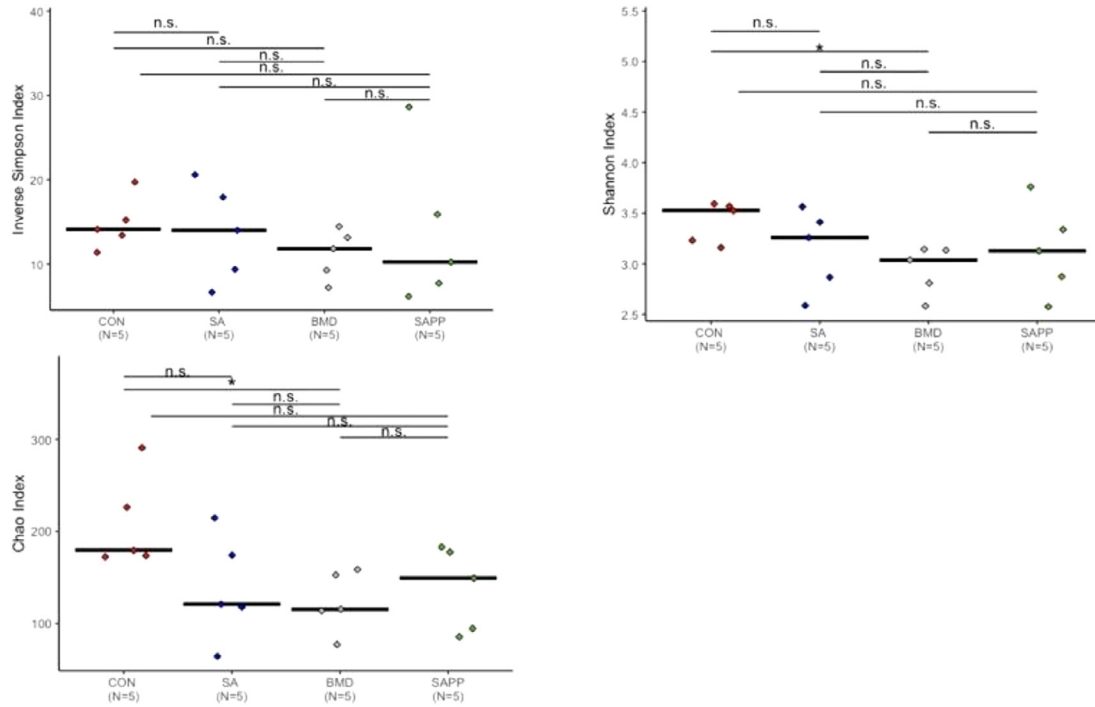


Figure 3. Alpha diversity analysis illustrating evenness, richness and diversity measures of cecal microbiome of chickens on d 35 of receiving a diet containing 0.15% bacitracin methylene disalicylate (BMD) at (150 g/ton) during the growling phase: starter (d 1 to 10 of age), grower (d 11 to 24 of age), and finisher (d 25 to 35 of age), or a diet containing 0.05% salinomycin (SA) at (50 g/ton) during the growling phase from d 1 to 35 of age, or a diet containing 0.05% salinomycin from d 1 to 35 of age and had access to medicated water containing 20 grams of penicillin G potassium (Pot-Pen)/100 liter of drinking water (SA + PP) from d 19 to 24 of age, or did not receive any medicated feed or water throughout the entire experiment (a negative control group). * indicates a significant difference at the 0.05 level between CON, SA, SAPP, and BMC that were compared. n.s. indicate a no significant difference at less than 0.05 between CON, SA, SAPP, and BMD that were compared.

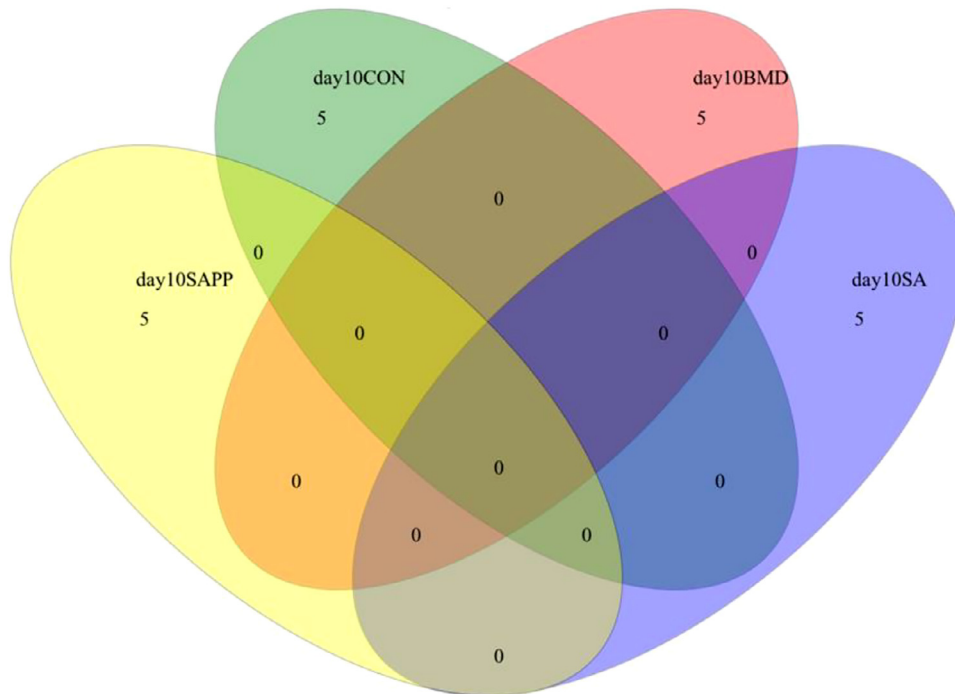


Figure 4. Venn diagram illustrates to compare the microbiome richness shared among chickens on d 10 of receiving a diet containing 0.15% bacitracin methylene disalicylate (BMD) at (150 g/ton) during the growling phase: starter (d 1 to 10 of age), grower (d 11 to 24 of age), and finisher (d 25 to 35 of age), or a diet containing 0.05% salinomycin (SA) at (50 g/ton) during the growling phase from d 1 to 35 of age, or a diet containing 0.05% salinomycin from d 1 to 35 of age and had access to medicated water containing 20 grams of penicillin G potassium (Pot-Pen)/100 liter of drinking water (SA+PP) from d 19 to 24 of age, or did not receive any medicated feed or water throughout the entire experiment (a negative control group).

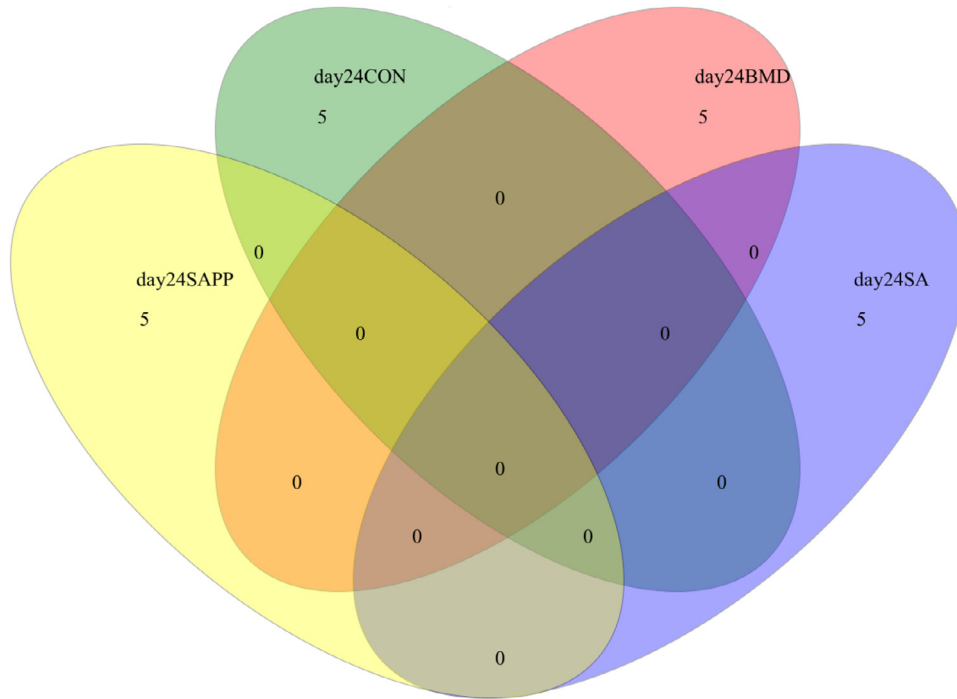


Figure 5. Venn diagram illustrates to compare the microbiome richness shared among chickens on d 24 of receiving a diet containing 0.15% bacitracin methylene disalicylate (BMD) at (150 g/ton) during the growning phase: starter (d 1 to 10 of age), grower (d 11 to 24 of age), and finisher (days 25 to 35 of age), or a diet containing 0.05% salinomycin (SA) at (50 g/ton) during the growning phase from d 1 to 35 of age, or a diet containing 0.05% salinomycin from d 1 to 35 of age and had access to medicated water containing 20 grams of penicillin G potassium (Pot-Pen)/100 liter of drinking water (SA + PP) from d 19 to 24 of age, or did not receive any medicated feed or water throughout the entire experiment (a negative control group).



Figure 6. Venn diagram illustrates to compare the microbiome richness shared among chickens on d 35 of receiving a diet containing 0.15% bacitracin methylene disalicylate (BMD) at (150 g/ton) during the growning phase: starter (d 1 to 10 of age), grower (d 11 to 24 of age), and finisher (d 25 to 35 of age), or a diet containing 0.05% salinomycin (SA) at (50 g/ton) during the growning phase from day 1 to 35 of age, or a diet containing 0.05% salinomycin from d 1 to 35 of age and had access to medicated water containing 20 grams of penicillin G potassium (Pot-Pen)/100 liter of drinking water (SA + PP) from d 19 to 24 of age, or did not receive any medicated feed or water throughout the entire experiment (a negative control group).

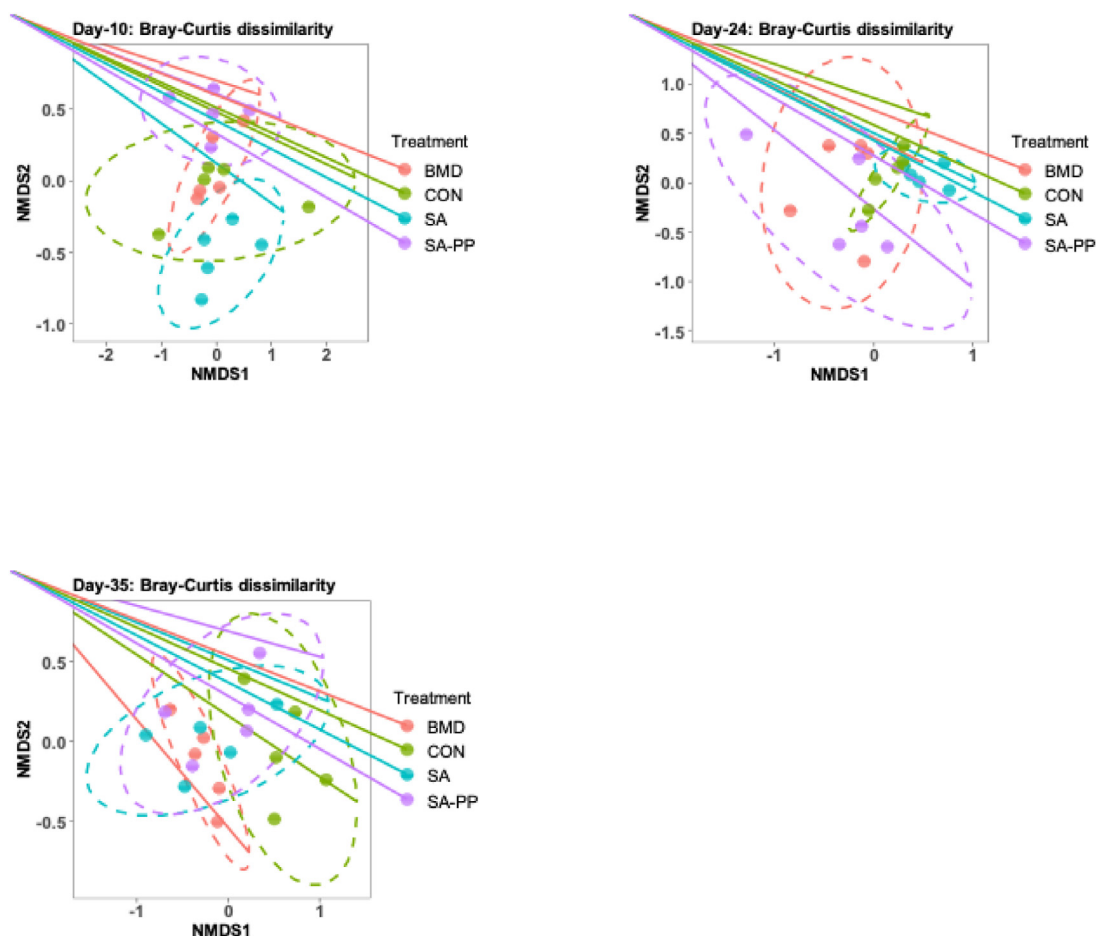


Figure 7. Nonmetric multidimensional scaling (NMDS) plot illustrating the chicken cecal microbiome beta-diversity on d 10, 24, and 35 following treatment with either a diet containing 0.15% bacitracin methylene disalicylate (BMD) at (150 g/ton) during the growling phase: starter (d 1 to 10 of age), grower (d 11 to 24 of age), and finisher (d 25 to 35 of age), or a diet containing 0.05% salinomycin (SA) at (50 g/ton) during the growling phase from d 1 to 35 of age, or a diet containing 0.05% salinomycin from d 1 to 35 of age and had access to medicated water containing 20 grams of penicillin G potassium (Pot-Pen)/100 liter of drinking water (SAPP) from d 19 to 24 of age, or did not receive any medicated feed or water throughout the entire experiment (a negative control group).

On d 35, the cecal microbial community structure of the SA, BMD, and SAPP groups varied significantly from that of the control group (Table 4). The microbial structure of the BMD-treated group was different from that of the SA- and SAPP-treated groups (Table 4).

Table 4. ANOVA *P*-value of treatment groups on d 10, 24, and 35 (iterations = 1000).

	CON	SA	BMD	SA+PP
CON ^a -D10	-	-	-	-
CON-D24	-	-	-	-
CON-D35	-	-	-	-
SA ^b -D10	0.003	-	-	-
SA-D24	0.003	-	-	-
SA-D35	0.01	-	-	-
BMD ^c -D10	0.40	0.16	-	-
BMD-D24	0.50	0.12	-	-
BMD-D35	0.008	0.06	-	-
SA-PP ^d -D10	0.15	0.005	0.26	-
SA-PP-D24	0.12	0.015	0.86	-
SA-PP-D35	0.06	0.31	0.04	-

^aCON is control treatment.

^bSA is salinomycin treatment.

^cBMD is bacitracin methylene disalicylate.

^dSA-PP is salinomycin plus penicillin G potassium.

Differential Enrichment of the Cecal Microbiome

Differences in the cecal microbiome of the antibiotic-treated and control chickens on d 10, 24, and 35 are shown in Figures 8–10. At the phylum level, on d 10, 24, and 35, the most abundant phylum between all treatments was Firmicutes (Figure 11). Proteobacteria was the most abundant phylum in the SA treatment group on d 10 (29.5%), 24 (15.3%), and 35 (8.0%; Figure 11). Actinobacteria proportion was higher in the control groups compared to other treatment groups on Day 10 (Figure 11). On d 35, the relative abundance of phylum Campilobacterota was higher in the BMD-treated chickens in comparison with other treatments (Figure 11). DESeq2 analysis was used to compare differential enrichment of the cecal microbiome of the antibiotic-treated and control chickens (Love et al., 2014; Figures 12, 14 and 15). On d 10, the cecal microbiome of the SA group was enriched with genera *Eisenbergiella*, *Vicivallaceae* *ge*, and *Incertae Sedis* and family *Enterobacteriaceae*. The most abundant genus in the SAPP group was *Anaerofilum* (Figure 12). RF39 *ge* was also found to be more abundant in SA and BMD treatment groups on

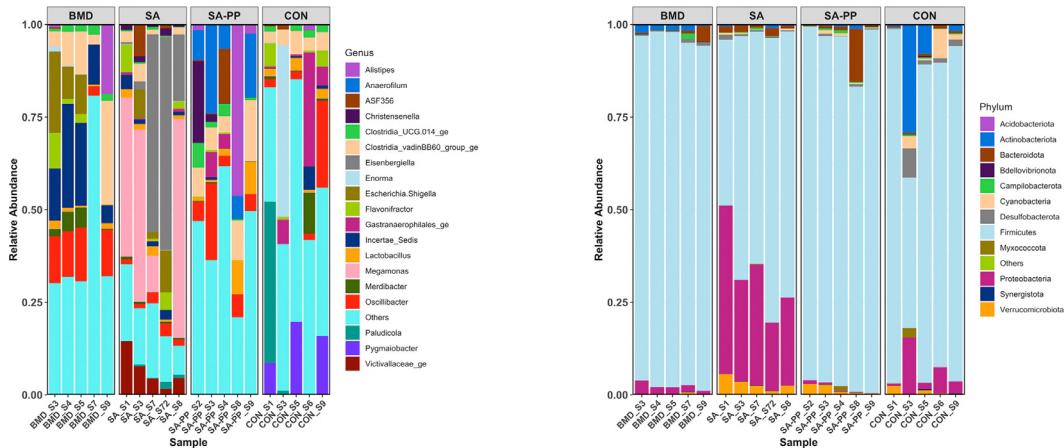


Figure 8. Differences in the cecal microbiome of the antibiotic-treated and control chickens on d 10 following treatment with either a diet containing 0.15% bacitracin methylene disalicylate (BMD) at (150 g/ton) during the growning phase: starter (d 1 to 10 of age), grower (d 11 to 24 of age), and finisher (d 25 to 35 of age), or a diet containing 0.05% salinomycin (SA) at (50 g/ton) during the growning phase from day 1 to 35 of age, or a diet containing 0.05% salinomycin from d 1 to 35 of age and had access to medicated water containing 20 grams of penicillin G potassium (Pot-Pen)/100 liter of drinking water (SAPP) from d 19 to 24 of age, or did not receive any medicated feed or water throughout the entire experiment (a negative control group). (A) Each bar represents the relative abundance of each bacterial taxa of chicken at phylum and genus level.

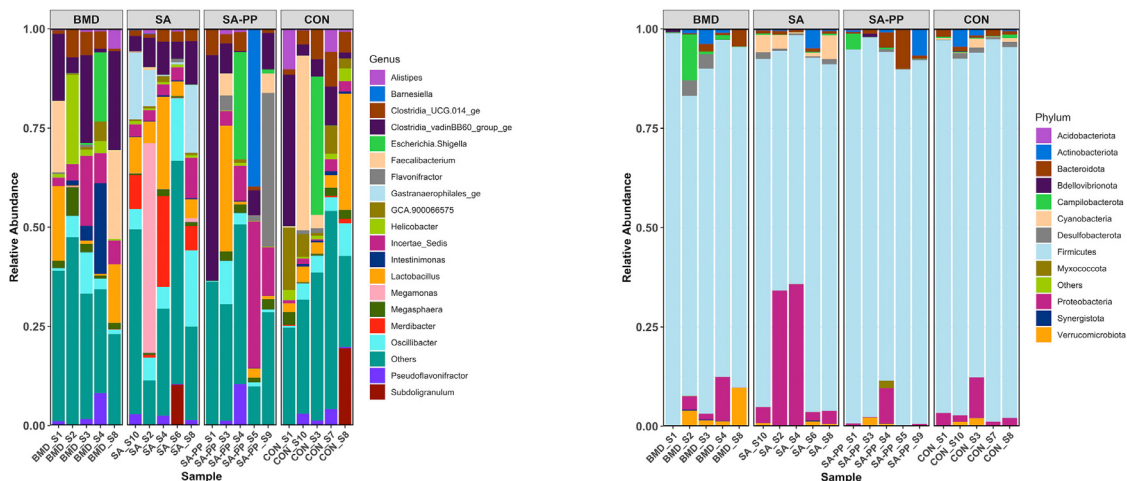


Figure 9. Differences in the cecal microbiome of the antibiotic-treated and control chickens on d 24 following treatment with either a diet containing 0.15% bacitracin methylene disalicylate (BMD) at (150 g/ton) during the growning phase: starter (d 1 to 10 of age), grower (d 11 to 24 of age), and finisher (d 25 to 35 of age), or a diet containing 0.05% salinomycin (SA) at (50g/ton) during the growning phase from d 1 to 35 of age, or a diet containing 0.05% salinomycin from d 1 to 35 of age and had access to medicated water containing 20 grams of penicillin G potassium (Pot-Pen)/100 liter of drinking water (SAPP) from d 19 to 24 of age, or did not receive any medicated feed or water throughout the entire experiment (a negative control group). (A) Each bar represents the relative abundance of each bacterial taxa of chicken at phylum and genus level.

d 10 (Figure 12). On the other hand, the SA and SAPP treatments resulted in a significant decrease in the unclassified Firmicutes (Figure 12). The relative abundance of Gram-positive and -negative bacteria on d 10 post-hatch was higher in the SAPP treatment and control groups (Figure 13).

On d 24, the cecal microbiome of the SA treatment and control groups was dominated with the family *Ruminococcaceae*, a butyrate-producing bacterium (Meehan and Beiko, 2014; Figure 14). The SA treatment group had higher relative abundance of genus *Gastranaerophilales ge*, *Merdibacter* and *Oscillospirales ge* compared to other treatment groups (Figure 14). There was not significant difference in the relative abundance of Gram-positive and -negative bacteria between the control group and SA, BMD, and SAPP treatment groups on d 24 (Figure 15).

On d 35, the cecal microbiome of the BMD group was significantly enriched with the genus *Helicobacter* (Figure 16), while the control group was enriched with the unclassified either Gram-positive or Gram-negative bacteria (Figure 16). The SA group had high abundance of unclassified class Bacilli (Figure 16). The cecal microbiome of the control group had significantly higher number of unclassified Gram-positive and -negative bacteria on d 35 (Figure 17).

DISCUSSION

In recent years, there has been extensive research on the mechanisms of action of AGPs, particularly their role in the modulation of gut microbiome composition (Engberg et al., 2000; Pourabedin et al., 2014;

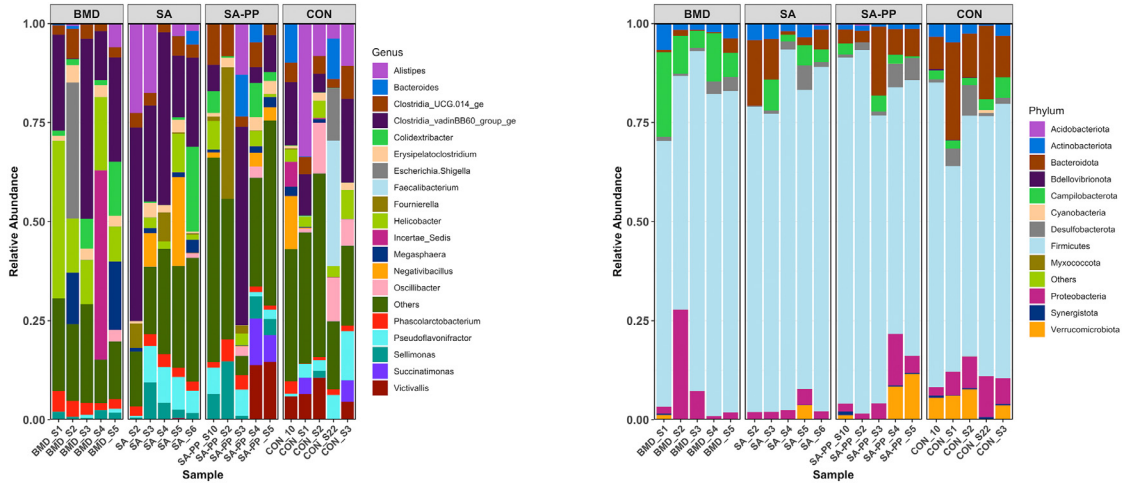


Figure 10. Differences in the cecal microbiome of the antibiotic-treated and control chickens on d 35 following treatment with either a diet containing 0.15% bacitracin methylene disalicylate (BMD) at (150 g/ton) during the growning phase: starter (d 1 to 10 of age), grower (d 11 to 24 of age), and finisher (d 25 to 35 of age), or a diet containing 0.05% salinomycin (SA) at (50g/ton) during the growning phase from d 1 to 35 of age, or a diet containing 0.05% salinomycin from d 1 to 35 of age and had access to medicated water containing 20 grams of penicillin G potassium (Pot-Pen)/100 liter of drinking water (SAPP) from d 19 to 24 of age, or did not receive any medicated feed or water throughout the entire experiment (a negative control group). (A) Each bar represents the relative abundance of each bacterial taxa of chicken at phylum and genus level.



Figure 11. Heatmap of relative abundance of the phylum-level changes in the cecal microbiome of the antibiotic-treated and control chickens on d 10, 24, and 35 following treatment with either a diet containing 0.15% bacitracin methylene disalicylate (BMD) at (150 g/ton) during the growning phase: starter (d 1 to 10 of age), grower (d 11 to 24 of age), and finisher (d 25 to 35 of age), or a diet containing 0.05% salinomycin (SA) at (50 g/ton) during the growning phase from day 1 to 35 of age, or a diet containing 0.05% salinomycin from d 1 to 35 of age and had access to medicated water containing 20 grams of penicillin G potassium (Pot-Pen)/100 liter of drinking water (SAPP) from d 19 to 24 of age, or did not receive any medicated feed or water throughout the entire experiment (a negative control group).

Costea et al., 2017; Proctor and Phillips 2019; Robinson et al., 2019). Despite the general consensus that AGPs can change gut microbial composition either by enhancing or limiting the growth of certain microbial species (Broom, 2017; Torok et al., 2011), there is scarcity of information about the effects of therapeutic doses of antibiotics on richness (number of bacterial species) and evenness (relative distribution) of microbial

communities in the chicken gut. As a case in point, while some studies demonstrated that supplementation of diet with AGPs such as avilamycin (Choi et al., 2018) and zinc bacitracin (Díaz Carrasco et al., 2018) decreases the alpha diversity index of the cecal microbiome, other studies revealed that their supplementation has no major effects (Danzeisen et al., 2011; Crisol-Martínez et al., 2017; Proctor and Phillips, 2019). These

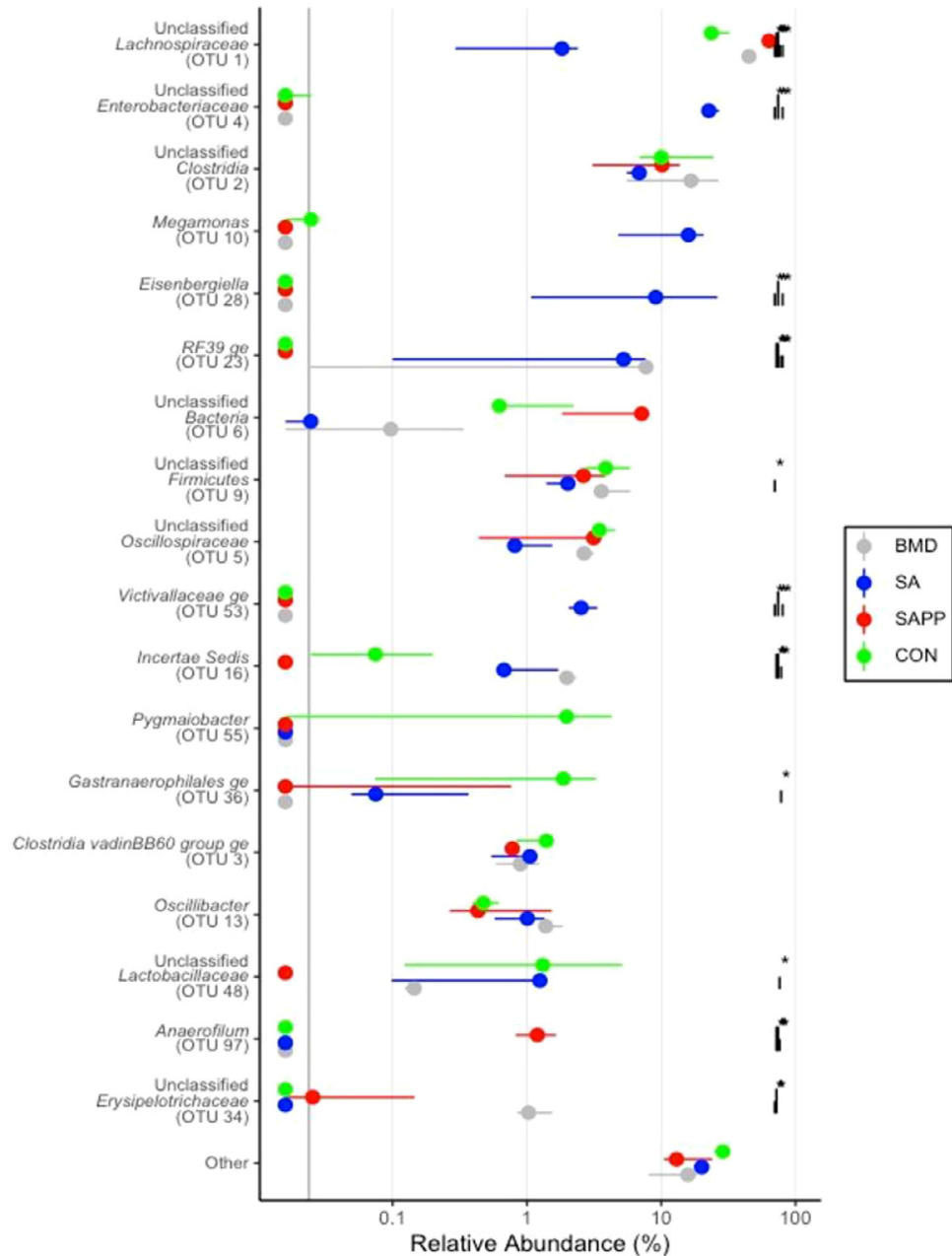


Figure 12. DESeq2 analysis illustrating differential enrichment of the cecal microbiome of the antibiotic-treated and control chickens on 10 following treatment with either a diet containing 0.15% bacitracin methylene disalicylate (BMD) at (150 g/ton) during the growning phase: starter (d 1 to 10 of age), grower (d 11 to 24 of age), and finisher (d 25 to 35 of age), or a diet containing 0.05% salinomycin (SA) at (50 g/ton) during the growning phase from d 1 to 35 of age, or a diet containing 0.05% salinomycin from day 1 to 35 of age and had access to medicated water containing 20 grams of penicillin G potassium (Pot-Pen)/100 liter of drinking water (SAPP) from d 19 to 24 of age, or did not receive any medicated feed or water throughout the entire experiment (a negative control group). | indicates a comparison between treatments. * indicates a significant difference at less than 0.05 between CON, SA, SAPP, and BMC that were compared.

conflicting data can be attributed to several environmental and experimental factors, including animal age, diets, stressors, and PCR biases (Hume et al., 2003; Yin et al., 2010; Torok et al., 2011; Zou et al., 2018).

In the present study, on d 10, the evenness and diversity of the cecal microbiome were decreased by BMD, SA, and SAPP treatments, while no such changes were observed on d 24 and 35. In addition, supplementation of the diet with these antibiotics decreased species richness compared to the control group on d 24 and 35. This could be attributed to their inhibitory or bactericidal effects against some Gram-positive bacteria which are

known to dominate the gut microbiome of chickens during the first week of life (Ballou et al., 2016). In fact, the cecal microbiome of newly hatched chickens is initially dominated by Gram-negative bacteria, which shifts during the first week of age to Gram-positive bacteria (Ballou et al., 2016) and as chickens age, their cecal microbial community changes to a more stable and complex population (Hume et al., 2003; Danzeisen et al., 2011; Torok et al., 2011; Glendinning et al., 2019). The results of the present study are consistent with a previous study by Chen et al. (2020), in which supplementation of the diet with virginiamycin decreased the Chao

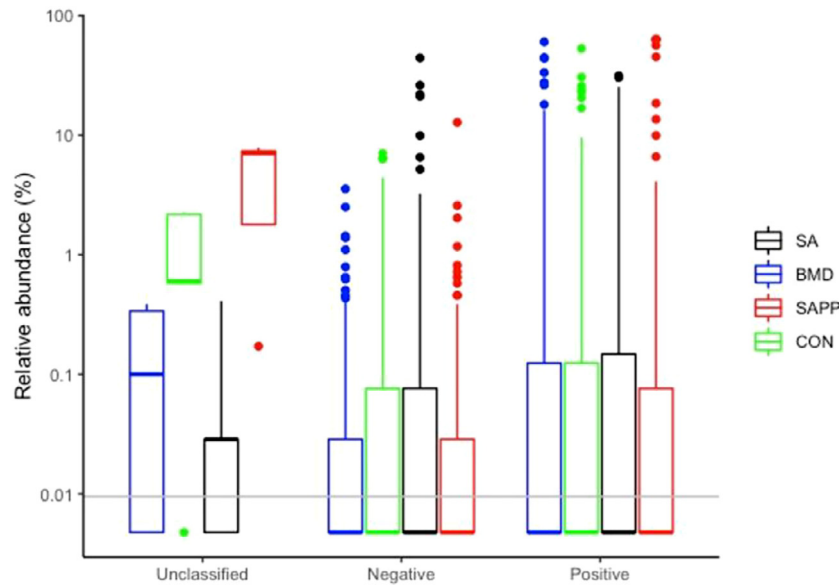


Figure 13. Differences in the cecal Gram-positive and negative microbiome of the antibiotic-treated and control chickens on d 10 following treatment with either a diet containing 0.15% bacitracin methylene disalicylate (BMD) at (150 g/ton) during the growling phase: starter (d 1 to 10 of age), grower (d 11 to 24 of age), and finisher (d 25 to 35 of age), or a diet containing 0.05% salinomycin (SA) at (50 g/ton) during the growling phase from d 1 to 35 of age, or a diet containing 0.05% salinomycin from d 1 to 35 of age and had access to medicated water containing 20 grams of penicillin G potassium (Pot-Pen)/100 liter of drinking water (SAPP) from d 19 to 24 of age, or did not receive any medicated feed or water throughout the entire experiment (a negative control group).

index of the cecal microbiome. In another study, while supplementation with ionophores (monensin and salinomycin) demonstrated a profound impact on richness and evenness of bacteria in the cecal microbiome of chickens compared to classic antibiotics including bacitracin methylene disalicylate, tylosin, and virginiamycin (Robinson et al., 2019), our results showed that BMD, SAPP, and SA treatments have comparable effects on microbial richness and evenness of the cecal microbiome.

Further, the results of Shannon analyses indicated that treatment with SAPP significantly decreased the diversity of the cecal microbiome, while treatment with BMD marginally decreased the diversity of the cecal microbiome, compared to the control group. Our results revealed that therapeutic levels of either antibiotics or ionophore induced significant alterations in the cecal microbial diversity compared to the subtherapeutic levels of these compounds. For instance, Robinson et al. (2019) observed that subtherapeutic concentrations of BMD, tylosin, and virginiamycin had no significant effects on the diversity of the chicken cecal microbiome. A possible explanation for this might be the below minimum inhibitory concentrations (sub-MIC) effects for the antibiotics used (Broom, 2017).

With respect to their effect on the cecal microbial composition, previous studies have shown that AGPs and ionophores differentially affect the cecal microbial communities (Broom, 2017; Robinson et al., 2019). Consistent with the results of Torok et al. (2011), dietary supplementation with SA resulted in enrichment of family *Enterobacteriaceae*, which includes many potentially pathogenic bacteria, such as *Escherichia coli*, *Shigella*, *Salmonella*, and *Klebsiella*, in the cecal microbiome on d 35. The family *Enterobacteriaceae* constitutes less than

1% of healthy intestinal microbiome (He et al., 2020). Although their presence is important for maintaining immunological balance in the intestine (Scarpa et al., 2011), the increased abundance of members of this family may result in economically significant enteric diseases including mucosal ulceration (He et al., 2020).

On d 10, the family *Victivallaceae* and *Erysipelotrichaceae* and the genus *Eisenbergiella* were most dominant in chickens fed SA. Families *Victivallaceae* and *Erysipelotrichaceae* are associated with improving feed conversion ratios in poultry while the genus *Incertae Sedis* has been associated with poor feed conversion ratios (Singh et al., 2012; Moore and Stanley et al., 2016). Family *Victivallaceae*, which belongs to the phylum Verrucomicrobiota, is a normal bacterial inhabitant in the human gut and a higher abundance of this family was observed in stool samples of patients with inflammatory bowel disease (IBD) (Bamola et al., 2017). Furthermore, SA treatment increased the relative abundance of genus *Eisenbergiella*, which belongs to the family *Lachnospiraceae*. Despite *Eisenbergiella*'s beneficial role in promoting gut health through expressing enzymes that mediate the production of butyrate, lactate, acetate and succinate (Amir et al., 2014), a positive correlation was observed between the abundance of genus *Eisenbergiella* and *Salmonella enterica* serovar Typhimurium in the gut microbiome (Khan and Chousalkar, 2020). Robinson et al. (2019) have also observed that while supplementation of chicken diets with antibiotics, such as bacitracin methylene disalicylate, tylosin and virginiamycin, and ionophores, such as monensin and salinomycin, enriched the cecal microbiome with members of the family *Ruminococcaceae*, they significantly reduced the abundance of *Lachnospiraceae* species.

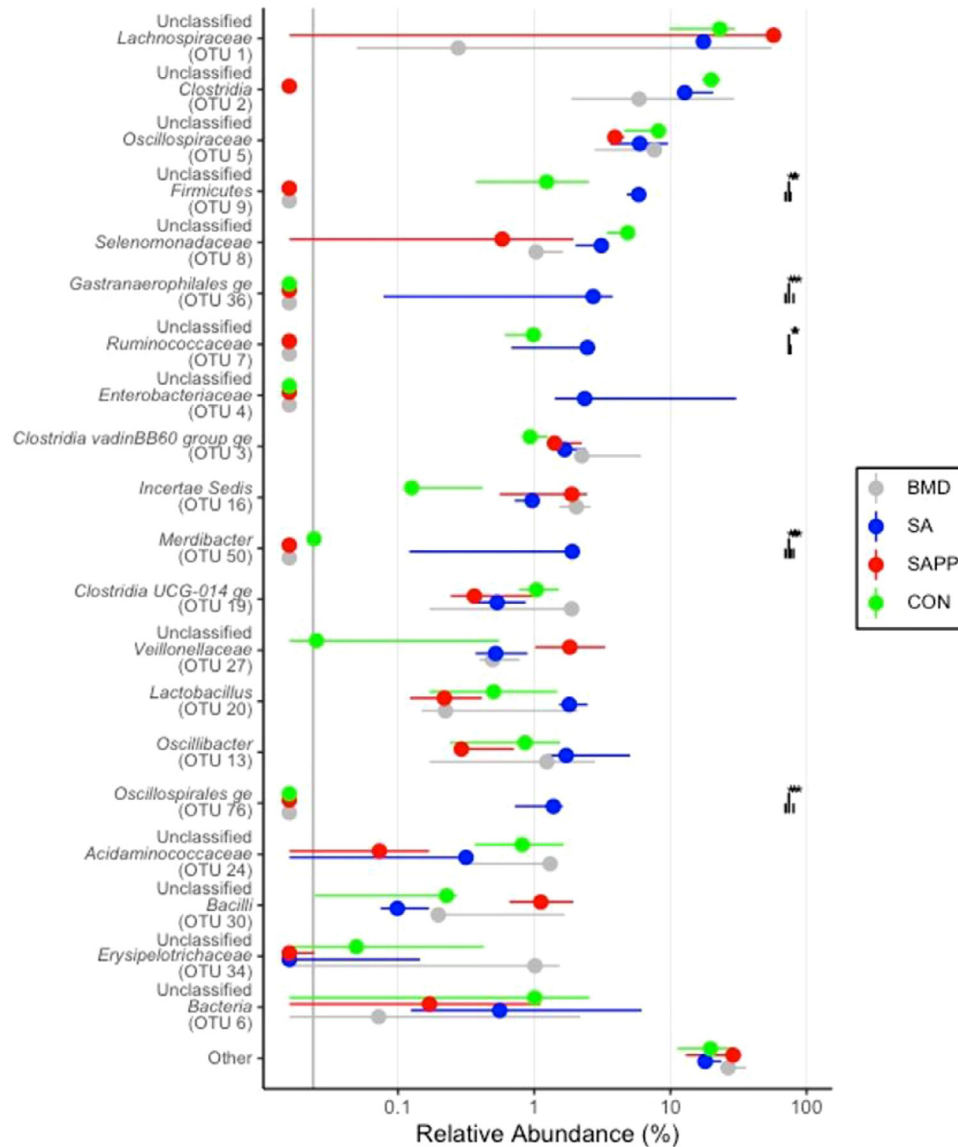


Figure 14. DESeq2 analysis illustrating differential enrichment of the cecal microbiome of the antibiotic-treated and control chickens on d 24 following treatment with either a diet containing 0.15% bacitracin methylene disalicylate (BMD) at (150 g/ton) during the growning phase: starter (d 1 to 10 of age), grower (d 11 to 24 of age), and finisher (d 25 to 35 of age), or a diet containing 0.05% salinomycin (SA) at (50 g/ton) during the growning phase from d 1 to 35 of age, or a diet containing 0.05% salinomycin from d 1 to 35 of age and had access to medicated water containing 20 grams of penicillin G potassium (Pot-Pen)/100 liter of drinking water (SAPP) from d 19 to 24 of age, or did not receive any medicated feed or water throughout the entire experiment (a negative control group). | indicates a comparison between treatments. * indicates a significant difference at less than 0.05 between CON, SA, SAPP, and BMC that were compared.

Supplementation of SAPP has been shown to increase the abundance of genus *Anaerofilum* which belongs to family *Ruminococcaceae* (Lee et al., 2017). A previous study by Danzeisen and colleagues has demonstrated that administration of coccidiostats, such as monensin and monensin/virginiamycin or tylosin, increases the abundance of *Anaerofilum* (Danzeisen et al., 2011). Furthermore, SAPP treatment also decreased the relative abundance of *Incertae Sedis* belonging to the family *Ruminococcaceae* and the abundance of family *Lactobacillaceae*, which are both known as butyrate-producing bacteria and have been previously shown to play a crucial role in improving feed efficiency (Meehan and Beiko, 2014; De Cesare et al., 2019). We also noted that on d 24, the cecal microbiome of SA-treated chickens and those in the control groups was enriched with family

Ruminococcaceae which belongs to class Clostridia, whose members are known to produce butyrate that possesses anti-inflammatory activities and contributes to the intestinal development of chickens (Onrust et al., 2015).

Experimental evidence indicates that butyrate produced by members of the phylum Firmicutes, during the early stage of the chicken life, is crucial for intestinal cell growth (Polansky et al., 2016). In view of this, the dominance of butyrate-producing bacteria and those expressing enzymes that are involved in butyrate production in the cecal microbiome of SA-, BMD-, and SAPP-treated chickens is indicative of the growth promoting role of these antibiotics.

The genus *Gastranaerophilales ge*, belonging to phylum Cyanobacteria, was most abundant in the SA

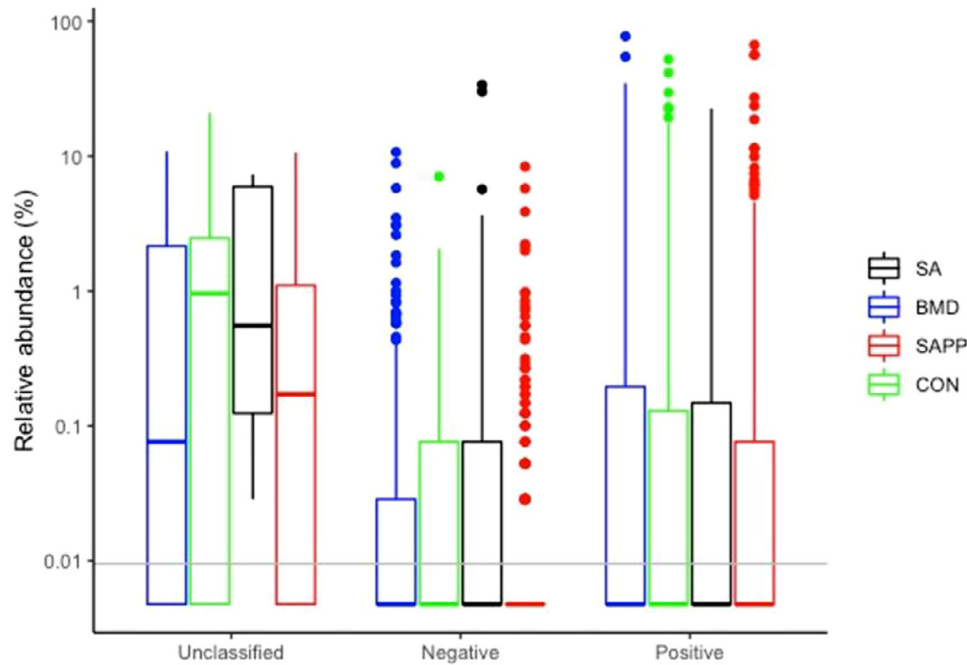


Figure 15. Differences in the cecal Gram-positive and negative microbiome of the antibiotic-treated and control chickens on d 24 following treatment with either a diet containing 0.15% bacitracin methylene disalicylate (BMD) at (150 g/ton) during the growling phase: starter (d 1 to 10 of age), grower (d 11 to 24 of age), and finisher (d 25 to 35 of age), or a diet containing 0.05% salinomycin (SA) at (50 g/ton) during the growling phase from d 1 to 35 of age, or a diet containing 0.05% salinomycin from day 1 to 35 of age and had access to medicated water containing 20 grams of penicillin G potassium (Pot-Pen)/100 liter of drinking water (SAPP) from d 19 to 24 of age, or did not receive any medicated feed or water throughout the entire experiment (a negative control group).

treatment group compared to BMD, SAPP and control groups. It is well documented that the abundance of genus *Gastranaerophilales ge* positively correlates with the average daily gain and some aspects of immune response in birds (Li et al., 2020). Recent studies have shown that members of class Gammaproteobacteria were associated with metabolic disorders and intestinal inflammation (Abaidullah et al., 2019; Gorecki et al., 2019). The birds treated with SA had a higher abundance of genus *Merdibacter*, belonging to class bacilli, in comparison with other treatments. *Merdibacter* facilitates the release of energy from food by secreting enzymes that are involved in the pentose phosphate pathways in the microbiome of human intestine (Anani et al., 2019). SA treatment also resulted in an increase in the relative abundance of genus *Oscillospirales ge* in the cecal microbiome. Adewole and Akinyemi et al. (2021) demonstrated that birds fed with high or normal energy diets treated with BMD had high abundance of genus *Oscillospirales ge* which was shown to negatively correlate with the population of the pathogenic bacteria, including *Streptococcus* (Adewole and Akinyemi et al., 2021).

Contrary to a previous observation by Danzeisen et al. (2011) that treatment with monensin and monensin/virginiamycin and monensin/tylosin decreases the abundance of Bacilli on d 7, 14, and 35 age of old, our results revealed that the cecal microbiome of chickens treated with SA had higher relative abundance of class Bacilli compared to the BMD, SAPP and control groups on d 35. These different effects may be attributed to their mode of action in disturbing cation transport

across the cell membrane of bacteria; salinomycin stimulates K^+ and Na^+ ions transportation across the cytoplasmic membrane, while monensin stimulates Na^+ and H^+ ions (Robinson et al., 2019).

Our study demonstrated a significant reduction in the abundance of family *Lactobacillaceae* in the cecal microbiome of SAPP-treated chickens and a dominance of this family in the cecal microbiome of SA- and BMD-treated and control chickens on d 10. Robinson et al. (2019) observed a depletion of genus *Lactobacillus* in the chicken cecal microbiome on d 14 following treatment with antibiotics, including bacitracin methylene disalicylate, tylosin and virginiamycin, and ionophores, including monensin and salinomycin. On the other hand, other studies have demonstrated that supplementation of chickens with SA or BMD decreases the number of *Lactobacillus* in the chicken intestinal microbiome (Engberg et al., 2000; Crisol-Martínez et al., 2017). To better interpret the variability in these data, comparative metagenomics studies are required to investigate the dynamic changes of the gut microbiome in response to different concentrations of antibiotics in age-matched chickens.

Overall, it appears that dietary supplementation of therapeutic levels of BMD, SA, and SAPP to chickens profoundly affected diversity and community composition of the cecal microbiome as demonstrated by depletion of several classes of beneficial bacteria, including Bacilli (Elshaghabee et al., 2017; Anani et al., 2019), Clostridia (Lopetuso et al., 2013; Onrust et al., 2015), and Actinobacteria (Binda et al., 2018), which all play a pivotal role in maintaining gut health.

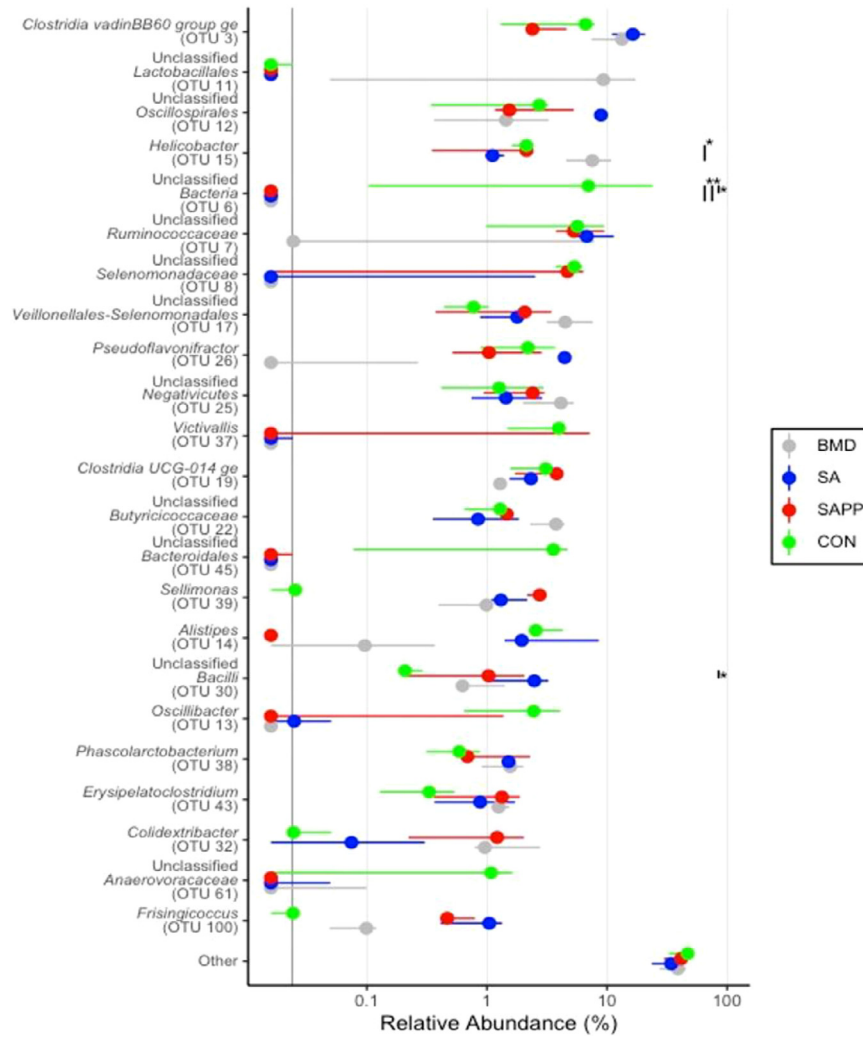


Figure 16. DESeq2 analysis illustrating differential enrichment of the cecal microbiome of the antibiotic-treated and control chickens on d 35 following treatment with either a diet containing 0.15% bacitracin methylene disalicylate (BMD) at (150 g/ton) during the growning phase: starter (days 1 to 10 of age), grower (d 11 to 24 of age), and finisher (d 25 to 35 of age), or a diet containing 0.05% salinomycin (SA) at (50 g/ton) during the growning phase from d 1 to 35 of age, or a diet containing 0.05% salinomycin from d 1 to 35 of age and had access to medicated water containing 20 grams of penicillin G potassium (Pot-Pen)/100 liter of drinking water (SAPP) from d 19 to 24 of age, or did not receive any medicated feed or water throughout the entire experiment (a negative control group). | indicates a comparison between treatments. * indicates a significant difference at less than 0.05 between CON, SA, SAPP, and BMC that were compared.

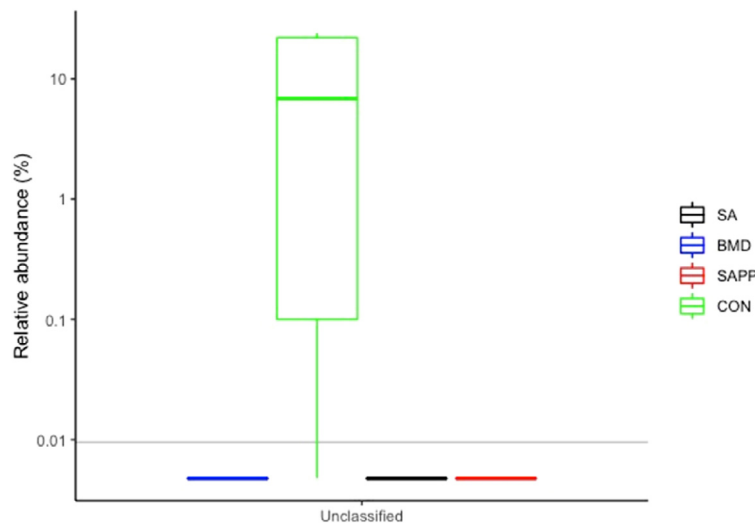


Figure 17. Differences in the cecal Gram-positive and negative microbiome of the antibiotic-treated and control chickens on d 24 following treatment with either a diet containing 0.15% bacitracin methylene disalicylate (BMD) at (150 g/ton) during the growning phase: starter (d 1 to 10 of age), grower (days 11 to 24 of age), and finisher (d 25 to 35 of age), or a diet containing 0.05% salinomycin (SA) at (50 g/ton) during the growning phase from d 1 to 35 of age, or a diet containing 0.05% salinomycin from d 1 to 35 of age and had access to medicated water containing 20 grams of penicillin G potassium (Pot-Pen)/100 liter of drinking water (SAPP) from d 19 to 24 of age, or did not receive any medicated feed or water throughout the entire experiment (a negative control group).

Furthermore, supplementation with salinomycin has been shown to increase the abundance of pathogenic bacteria, including order *Gastranaerophilales* (Li et al., 2020), class *Gammaproteobacteria* (Gorecki et al., 2019; Abaidullah et al., 2019) and family *Enterobacteriaceae* (He et al., 2020) that have been previously shown to be associated with metabolic disorders and gastrointestinal diseases.

It is noteworthy that a few members of phyla *Bacteroidetes* and *Firmicutes*, particularly butyrate-producing bacteria, were enriched in the cecal microbiome of both antibiotic- and ionophore-treated chickens, albeit not at the same level of diversity and abundance as in the control chickens.

CONCLUSIONS

Dietary or water supplementation of chickens with therapeutic levels of antibiotics and ionophores has been shown to significantly alter the cecal microbial homeostasis during different stages of the chicken life. Given the important role of the gut microbiome in regulating host metabolism and immunity, these findings highlight the importance of prudent use of antibiotics in poultry production and suggest the need for in-feed or water supplementation of chickens with beneficial microbial consortia following treatment with these antibiotics to restore microbial richness and diversity in the gut microbiome. Further investigations are required to determine the functional changes in intestinal immune responses, metabolic activity of the gut microbiome and growth performance of the antibiotic-treated chickens.

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Availability of Data and Material: The raw amplicon sequencing data from this study is available in the NCBI Sequence Read Archive (SRA; <https://www.ncbi.nlm.nih.gov/sra/>) with the BioProject identifier PRJNA805262.

DISCLOSURES

The authors declare no conflict of interest.

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