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Pyrosequencing Analysis of Salinomycin and *Eimeria* spp. Challenge-Induced Changes in Broiler Cecal Microbial Communities

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Abstract: *Eimeria* spp. invade and damage the intestinal cell lining of broilers resulting in cell necrosis and secondary bacterial infections. The current work investigates the effect of anticoccidial agents, salinomycin in combination with Roxarsone and *Eimeria*-challenge on the composition of broiler cecal microflora. Three hundred and twenty day-old male Cobb broilers were among four treatment groups: NN (no salinomycin and no *Eimeria* challenge) and NC (no salinomycin plus *Eimeria* challenge) received basal diet with no salinomycin, while SN (salinomycin and no *Eimeria* challenge) and SC (salinomycin plus *Eimeria* challenge) received basal diet with salinomucin. Broilers in groups NC and SC were orally gavaged on d 28 with a mixed *Eimeria* spp. challenge. Body weight and *Eimeria* lesion scores were determined at d 35. Cecal bacterial DNA from broilers at day 28 and day 35 were subjected to 454 pyrosequencing of 16S rDNA for sequence identification. Relative percent abundance and richness of the identified taxa were analyzed. Salinomycin had significant influence on the total number of taxa ($p = 0.02$) and on cecal microbial community structure ($p = 0.002$). The mixed *Eimeria* challenge marginally affected the total number of taxa ($p = 0.06$) and the composition of microbial communities ($p = 0.09$). Broiler age, body weight and *Eimeria* lesion score had no significant effect on the cecal microbial communities. Results from this study indicate that pyrosequencing is effective in understanding the dynamics and functionality of cecal microbial communities in relation to anticoccidial treatment, *Eimeria* challenge and broiler performances.

Key words: Coccidiosis, salinomycin, cecal microbiome, pyrosequencing, redundancy analysis

INTRODUCTION

Broiler production is one of the fastest growing segments of the poultry industry around the world. The dynamics of poultry gastrointestinal microbial ecology is being studied with a goal of improving the quality of production (Apajalahti and Bedford, 1999; Apajalahti *et al*., 2004; Dibner and Richards, 2004; Zoetendal and Mackie, 2005). Studies have shown that diet, feed supplements, age and presence of intestinal pathogens such as *Eimeria* spp. influence the microbial community structure in the intestine of broilers (Hume *et al*., 2006; Lu *et al*., 2003; Oviedo-Rondón, 2006a,b, 2009). *Eimeria* spp., commonly called chicken coccidia, are prolific parasitic protozoans that infect the intestine of birds, leading to necrotic tissue and further resulting in necrotic enteritis caused by secondary bacterial challenges (McDougald and Fitz-coy, 2008; McDougald *et al*., 2008; McMullin, 2001; Williams, 2005). Additional efforts by the poultry industry have focused on developing feed additives which could control these pathogens along with improving broiler performance and positively modulating the intestinal microflora (Thomke and Elwinger, 1998; Verstegen and Williams, 2002).

It has been a standard practice in the poultry industry to use anti-coccidial agents as feed supplements to prevent, reduce, or eliminate the disease condition and enhance production. However, the type of feed additive, the dose, medium of application and many other factors cause a wide range of variability in their efficacy of *Eimeria* coccidiostats are classified into two types based on the mode of synthesis: chemicals and ionophores. Chemical coccidiostats are synthetically manufactured and are further classified by chemical composition and mode of action. A few commonly used chemical coccidiostats are diclazuril, dinitrocarbanilide, halofuginone and amprolium (McDougald and Fitz-Coy, 2008). Ionophoric coccidiostats are synthesized by microbial fermentation. This group of coccidiostat disrupts the transmembrane ion concentration gradients required for the survival of the parasite. Some of the ionophoric coccidiostats are narasin, monensin, lasalocid and salinomycin (Singla *et al*., 2007). There are some comparative studies on the efficacy of these coccidiostats in broilers (Ashraf *et al*., 2002; Brown, 2007; Duffy *et al*., 2005; Singla *et al*., 2007). Salinomycin has been proven as a more effective anti-coccidial than

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all other coccidiostats in terms of weight gain, Feed Conversion Ratio (FCR), oocyst count and reduction in mortality (Ashraf *et al*., 2002). In order to understand the key changes occurring in the intestine as a result of feed supplements, it is important to monitor the dynamics of gut microflora. It is additionally important to determine the vital roles that microbial communities play under conditions created by the presence of these feed additives and while establishing their functionality (Zoetendal and Mackie, 2005).

Studying the dynamics of the intestinal microbial communities has been a challenge due to the limitations and availability of bacterial culturing methods (Zoetendal and Mackie, 2005). Culture-independent molecular methods first applied in the early 1990s have allowed researchers to investigate the composition of intestinal microbial communities with greater depth and without much of the bias associated with culture-dependent methods. These molecular methods are based on the analysis of whole microbial communities using relative abundance of GC bases contained in genomic DNA, sequencing of clone libraries and/or electrophoresis fingerprinting techniques such as denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE). Analysis of data generated by these methods provides a snap-shot of the status and modifications of microbial communities. Some studies using these methods focused on monitoring succession and shifts of microbial communities in the cecum of birds under different management, nutritional and health conditions (Amit-Romach *et al*., 2004; Apajalahti and Bedford, 1999; Apajalahti *et al*., 2001, 2004; Guo *et al*., 2004; Hume *et al*., 2003, 2006; Oviedo-Rondón *et al*., 2006a; Van der Wielem *et al*., 2002). Among these studies, only a few have demonstrated the functionality of the presence and abundance of specific bacterial taxa with performance results. Previously, identification of specific bacterial communities was very difficult due to the limitations in sequencing methods. Recent technology developments have allowed researchers to overcome this problem. With the advent of pyrosequencing and bacterial tag-encoded FLX-titanium amplicon pyrosequencing approach (bTEFAP, Dowd *et al*., 2008a,b), a more comprehensive and relatively quantitative assessment of the dynamics of intestinal microbiome at detailed taxonomic resolution is made possible. The present study evaluates the effects of salinomycin on the dynamics of specific bacterial taxa of cecal microbial communities and the performance of broilers, during the periods before and after challenge with mixed *Eimeria* spp.

MATERIALS AND METHODS

Broiler husbandry and treatments: A total of 320, day-old male Cobb broilers were equally divided among four treatment groups: Groups NN (no salinomycin and no *Eimeria* challenge) and NC (no salinomycin plus *Eimeria* challenge) received basal diet with no salinomycin and groups SN (salinomycin and no *Eimeria* challenge) and SC (salinomycin plus *Eimeria* challenge) received basal diet supplemented with 60 g/ton of salinomycin (Bio-Cox®, Alpharma, Inc., Ft. Lee, NJ). Broilers in groups NC and SC were orally gavaged on d 28 with 0.2 mL of a mixed *Eimeria* spp. challenge (approximately 100,000 oocysts of *E. acervulina*, 60,000 oocysts of *E. tenella* and 40,000 oocysts of *E. maxima*). Broilers were reared in four, 1.5×3.0 m floor pens in a tunnel-ventilated facility, where challenged and non-challenged groups were separated by a footpath to reduce possible contamination. All broilers received the same corn-soybean meal diet formulated to meet the requirement of broiler chickens framed by the NRC (1994). The feed content of the diet was previously described by Oviedo-Rondón *et al*. (2005). Starter feed (basal diet with 50 g/ton of bacitracin methylene disalicylate (BMD®, Alpharma, Inc., Ft. Lee, NJ) and 45 g/ton roxarsone (3-Nitro-4-hydroxy phenyl arsonic acid; Alpharma, Inc.) was crumbled and fed from d 1 to d 18. Grower feed (basal diet with 25 g/ton of BMD and 22.5 g/ton of roxarsone) was pelletized and fed from d 19 to d 35. Clean water and feed were provided *ad libitum* throughout the study.

Data and sample collection: On d 28 and d 35, ten birds in each treatment group were randomly selected and weighed individually using a Doran® 8000XL digital scale. Each bird weight was recorded and used to calculate an average body weight for each treatment group. Each bird was then humanely euthanized by rapid cervical dislocation and examined for the presence of coccidiosis lesions. Lesion scoring was performed according to the Johnson and Reid (1970) method. Both ceca were aseptically removed from each bird immediately after the selected chickens were euthanized at d 28 (pre-challenge) and d 35 (post-challenge). Both ceca were placed in a sterile Whirl-Pak bag (Nasco, Modesto, CA) and immediately stored at -20°C until further processing. Three samples were randomly chosen from each treatment group on d 28 and d 35 for further processing. Therefore, a total of 18 samples, three from each treatment group on d 28 (NN and SN) and on d 35 (NN, NC, SN and SC), were processed for DNA extraction and pyrosequencing of 16S rDNA.

DNA extraction and 16S rRNA PCR: Contents DNA from a total of 18 chicken cecal samples, consisting of three samples from each treatment group on d 28 (NN and SN) and on d 35 (NN, NC, SN and SC) were extracted using the Maxwell® 16 Tissue DNA purification kit (Promega Corp., Madison, WI) with an automated DNA extractor Maxwell® 16 Tissue DNA Purification (Promega)

by following the manufacturer's protocol. Approximately, 50 mg of cecal contents ceca were used for DNA extraction. The quality and quantity of the isolated DNA was assessed using GeneQuant Pro (Amersham Biosciences Corp., Piscataway, NJ). After quantification, the DNA samples were stored at -80°C until further use. The stored DNA samples were assessed for the presence of 16S rDNA gene by performing polymerase chain reaction (PCR) specific for the amplification of 16S rDNA highly variable V3 region sequences. The PCR was performed in a 25 μL reaction volume with 12.5 μL of JumpStart™ REDTaq® ReadyMix™ (Sigma-Aldrich, Inc., St. Louis, MO), 1 μL (20 ng/μL) of Forward primer (5'-CCT ACG GGA GGC AGC AG-3') and 1 μL (20 ng/μL) of Reverse primer (5'-ATT ACC GCG GCT GCT GG-3') (Integrated DNA Technologies, Inc., Coralville, IA), 1 μL of DNA sample and deionized nuclease-free water to make up the reaction volume. The PCR reaction mixture was thermocycled in the PCR machine (Eppendorf Corp., Hauppauge, NY) using the following program: 97°C for 5 min; 40 cycles of 60°C for 1 min, 72°C from 1 min 20 sec and 95°C for 30 sec; 72°C for 5 min and hold at 4°C. The presence of a 200-bp PCR amplicon was verified using agarose gel electrophoresis with 1×TAE (40 mM TRIS, 20 mM acetic acid, 1 mM EDTA, pH 8.0) buffer on 1.2% agarose gel along with all purpose Hi-Lo DNA maker (Bionexus, Inc., Oakland, CA) at 90 V for 45 min.

Pyrosequencing: The sequencing of the 16S rDNA in the sample was performed after partial ribosomal amplification followed by Bacterial Tag-encoded FLX amplicon pyrosequencing as described by Dowd *et al*. (2008a). Briefly, the protocol involves the following steps. The 16S rDNA in the sample was amplified using universal 16S eubacterial primers 530F and 1100R. A secondary PCR was performed for 454 amplicon sequencing using specially designed fusion primers with different tag sequences: Linker A-tags-530F and Linker B-tags-1100R. After the secondary PCR, all products were purified using nanomagnetic beads. Then equal amounts of this dsDNA were mixed with an equal amount of DNA capture beads and the bound DNA was amplified by emulsion PCR. After bead recovery and enrichment, the bead-attached DNA was denatured and sequencing primers were annealed. Then the beads were placed in a PicoTitrePlate (one bead per well) along with sequencing reaction mixtures. Then sequencing was performed using the Genome Sequencer FLX system (F. Hoffmann-La Roche Ltd., Nutley, NJ) according to the manufacturer's protocol.

Sequence data analyses: Taxonomic assignment of sequences was performed with RDP Classifier 2.0 (Ribosomal Database Project Classifier 2.0; Wang *et al*., 2007). The RDP Classifier is commonly used to assign higher-order bacterial taxonomy to the 16S rRNA gene sequences. These taxa are assigned based on a naive Bayesian rRNA classifier. The sequences were classified according to the confidence levels calculated by RDP. The confidence cutoff value (>80%) was used as a criterion for the taxonomic assignment. The sequence was classified at genus level if the confidence value was >80%, otherwise it was assigned to higher level of taxonomy, for example family, unless the confidence was less than 80%. Relative percent abundance of taxa per sample was calculated to form a taxa matrix. The taxa matrix was imported into R statistical environment (Oksanen *et al*., 2010) version 2.9.1 (Free Software Foundation, Inc., Boston, MA, http://www.R-project.org) and the following analysis was performed. The species richness was expressed as total number of taxa found in a sample. The total number of taxa in each sample was calculated and a tiled scatterplot was generated using the lattice package. Differences between the total numbers of taxa in different treatment groups were assessed using an ANOVA. A $p<0.1$ is considered as significant in the analysis. However, $p<0.05$ is considered as highly significant and a value between $p>0.05$ and <0.1 is considered moderately significant. Hierarchical clustering and redundancy analysis (RDA) was performed on the taxa abundance data using the vegan package.

Bray-Curtis dissimilarity index for a pair of samples was calculated to assess the similarities between bacterial communities in the samples. Then, the hierarchical clustering of the samples was performed using average linking method of the calculated index. A barchart showing the relative percent abundance of each taxa in the sample was generated using the lattice package. To choose the appropriate method for performing multivariate analysis, detrended correspondence analysis (DCA) of the taxa abundance data (Hellinger transformed) was performed. This analysis determined a gradient less than 3 standard deviation units, which showed that linear method was appropriate (Ter Braak and Prentice, 1988). The effect of salinomycin and *Eimeria* challenge on species composition and its relationship with broiler performance parameters was determined using the redundancy analysis. The ordination plots were created using the BiodiversityR package. The significance of the amount of variation explained by the parameters (body weight of birds and coccidiosis lesion scores) was tested by variation partitioning analysis using Monte Carlo permutation tests (10,000 permutations; McArdle and Anderson, 2001).

RESULTS AND DISCUSSION

Average body weight for all sample birds (n = 10/ treatment) was not significantly different between

treatments on d 28 or d 42 (Table 1). Weights on d 28 and d 42 ranged from 1.43 to 1.49 kg and 2.81 to 3.17 kg, respectively. Coccidiosis lesion scores were minimal in birds examined on d 28 prior to *Eimeria* challenge. One out of the ten birds examined in the NN, NC and SN treatment groups exhibited *E. maxima* lesions that were scored as a +1. No *E. acervulina* or *E. tenella* lesions were observed in any treatment group on d 28. By d 42, all birds from the two challenged groups, NC and SC, exhibited *E. acervulina* and *E. maxima* lesions varying in severity from +1 to +3. The non-challenged groups, NN and SN, only exhibited a +1 *E. maxima* lesion score in one bird of each treatment group. *No E. tenella* lesions were observed for any treatments group throughout the study. Coccidiosis lesion scores demonstrated that our mixed *Eimeria* challenge simulated an infection in the challenged groups. We believe that the use of roxarsone in the diets led to the absence of any *E. tenella* infection in the challenged birds. This inference was in total agreement with the previous report by McDougald *et al*. (1992), who showed that roxarsone has significant effectiveness against *E. tenella*.

A total of 85,500 bacterial 16S rDNA sequences were obtained from the pyrosequencing of 18 cecal samples. Approximately 4,750 sequences were obtained per sample with an average length of 470 bp. Almost all of the sequences (99.9%) belonged to the kingdom Bacteria except one which belonged to the kingdom Archaea. As classification moved from the level of kingdom to genus, the number of taxa observed at each level progressively increased. Overall, 14 phyla (*Firmicutes* was dominant with 96.6%) and 203 genera (*Faecalibacterium* was dominant with 16.7% abundance) were observed. In the final data set of taxa abundance, a total of 211 unique taxa (out of which 96.2% were classified to genus level and 3.8% to family level) were observed in all the samples together. The major bacterial taxa *Lachnospiraceae* (17.1%), *Faecalibacterium* (16.7%) and *Papillibacter* (14.3%) observed at the genus level belonged to the phylum *Firmicutes*. Some of the beneficial (i.e., helping to establish conditions of benefit to the host organism) bacterial taxa observed at the genus level were *Lactobacillus* spp. (2.3%), *Lachnospiraceae* spp. (17.1%), *Faecalibacterium* spp. (16.7%), *Ruminococcus* and *Ruminococcaceae* spp. (6.2%), *Acetanaerobacterium* spp. (5.4%) and *Citrobacter* spp. (0.4%). Potential pathogenic bacterial taxa observed were *Bacteroides* spp. (0.05%), *Enterococcus* spp. (0.03%), *Salmonella* spp. (0.03%), *Shigella* spp. (1.2%) and *Streptococcus* spp. (0.4%). The relative abundance of these species varied with the type of treatment. The distribution of the total number of taxa by treatment group is shown in Fig. 1.

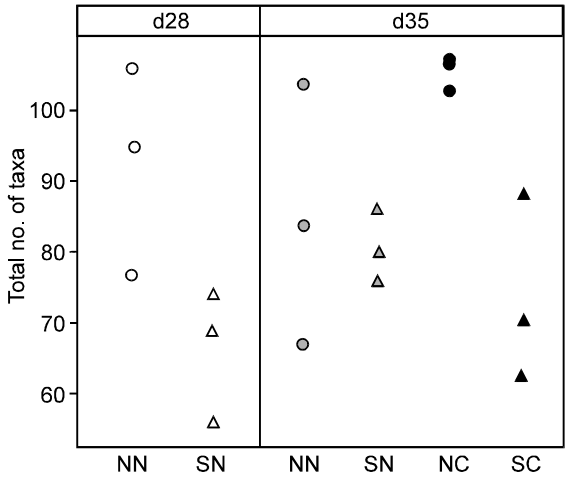

Fig. 1: Distribution of identified taxa among treatment groups. Cecal contents DNA were examined from each of three chicks per treatment group. Circles represent no salinomycin in feed. Triangles represent samples from broilers supplemented with salinomycin. Blank symbols represent pre-challenge (d 28), black symbols post-challenge (d 35) and gray symbols represent post-challenge control (d 35) broilers. The NN were given no salinomycin and no *Eimeria* challenge, SN was given salinomycin and no *Eimeria* challenge, NC was given no salinomycin and were given *Eimeria* challenge and SC were given salinomycin and *Eimeria* challenge

Table 1: Average values[1] of body weight and *Eimeria* lesion scores in broilers at d 28 and d 35

			Lesion Score[2]		
Day	Group*	BW (lb)	*E. acervulina*	*E. maxima*	*E. tenella*
28	NN	1.44±0.08	0	1±0.58	0
	SN	1.30±0.31	0	0	0
35	NN	2.34±0.05	0	0	0
	SN	1.91±0.13	0	1±0.33	0
	NC	2.06±0.26	3±0.33	2±0.33	0
	SC	2.15±0.17	3±0.33	1±0.33	0

*NN: No Salinomycin + No *Eimeria* challenge
SN: Salinomycin + No *Eimeria* Challenge
NC: No Salinomycin + *Eimeria* challenge
SC: Salinomycin + *Eimeria* challenge
[1]Values = Mean ± SE, n = 3
[2]Lesion scores on a scale of 0 to 4 (Johnson and Reid, 1970)

The highest number of taxa (107) was observed at seven days post-*Eimeria* challenge samples in the group NC given no salinomycin (Fig. 1). The least number of taxa (56) was found on d 28 in one chick in the pre-challenged SN group, which received salinomycin supplemented in their diet and no *Eimeria* challenge (Fig. 1). In general we observed smaller numbers of taxa in ceca of broilers which received

Table 2: P-values estimated from the linear model (R^2: 0.6338, P-value: 0.02014) of total number of taxa and the experimental variables day, treatment, and *Eimeria* challenge

Variable	Slope	P-value
35 day	-7.67	0.45 ^{NS}
Salinomycin	-26.33	0.02
Eimeria challenge	20.67	0.06
35 day: Salinomycin	22.00	0.14 ^{NS}
Salinomycin: *Eimeria* challenge	-28.00	0.06

^{NS} Not significant

salinomycin in their diet in both pre-challenged (d 28) and post-challenged (d 35) groups (Fig. 1). In groups not given salinomycin, the numbers of taxa in ceca did not significantly (p = 0.45) decrease with the aging of the broilers. Groups given the *Eimeria* challenge did experience a moderately significant (p = 0.06, Table 2) increase in the number of cecal bacterial taxa. Whereas in the salinomycin groups, the numbers of taxa did not significantly (p = 0.14) increase with an increase in broiler age and with *Eimeria* challenge. Two thirds of the treatment groups showed a moderate decrease (p = 0.06) in the number of taxa in the ceca (Table 2). Supplementation of salinomycin in feed significantly (p = 0.02) affected the total number of taxa in the ceca (Table 2). However, the numbers showed that aging of the broilers marginally decreased the number of cecal taxa in broilers which received no salinomycin (Fig. 1). Aging of broilers in the salinomycin groups resulted in a marginal increase in the numbers of taxa in the ceca (Fig. 1).

Hierarchical clustering analysis of taxon abundance data distinguished three major clusters: A, B and C as shown in Fig. 2. The clusters were the control group (Cluster A, NN from d 28 and NC from d 35), the post-challenge control group (Cluster B, NN and SN from d 35) and salinomycin group (Cluster C, SN from d 28 and SC from d 35). The clusters showed distinct grouping of samples into the no salinomycin cluster and the salinomycin cluster, with exception of the post-challenge control group (Cluster B, gray shaded samples). The dissimilarity index between the samples in Cluster C (salinomycin group) was high and the taxon abundance data in this cluster was different from other samples. In the post-challenge control groups (d 35 NN and SN), the dominant taxa were *Faecalibacterium* (29%), *Lachnospiraceae* (23.1%) and *Papillibacter* (24.8%) all at similar levels. *Ruminococcaceae* (9.2%) and *Acetanaerobacterium* (6.6%) were moderately high in the SN group, while *Sporobacter* (4.4%) was moderately high in the NN group. In the pre-challenge group (d 28, Blank symbols in Fig. 2), *Faecalibacterium* (41.3%) and *Ruminococcaceae* (10.2%) was observed in similar levels in both NN and SN groups. *Anaerotruncus* (9.6%), *Papillibacter* (18.5%) and *Lactobacillus* (22.7%) were high in the NN group, while *Lachnospiraceae* (47.1%), *Roseburia* (9.1%), *Syntrophococcus* (4.2%) and *Acetanaerobacterium* (4.4%) were high in SN group. In the post-challenge group (d 35, Black symbols in Fig. 2), *Papillibacter* (25.5%), *Peptococcus* (23.4%) and *Sporobacter* (5.3%) were high in the NC group, while *Acetanaerobacterium* (27.7%), *Faecalibacterium* (11.1%) and *Lachnospiraceae* (21.2%) were high in SC group. The hierarchical clustering of bacteria in samples from each group showed that the cluster group was not only based on the abundant taxa, but also was influenced by the presence and absence of specific taxa in the group.

Overall, 121 taxa were common to both salinomycin (SN and SC) and no salinomycin (NN and NC) groups. Approximately 60 taxa were specific for only the no-salinomycin groups, of which 14 taxa were specific for pre-challenge (d 28 NN), 5 for post-challenge (d 35 NN), 24 for post-challenge (d 35 NC) and the rest appeared in 2 or more groups. Approximately 30 taxa were specific for the salinomycin group, of which 5 taxa were specific for pre-challenge (d 28 SN), 6 for post-challenge (d 35 SN), 16 for post-challenge (d 35 SC) and the rest appeared in 2 or more groups. A total of 128 taxa were common in both challenged (NC and SC) and non-challenged (NN and SN) groups. Forty-three taxa were specific for the non-challenged groups of which 21 appeared only in the NN group and 12 only in the SN group. These 43 taxa appeared commonly in the d 28 and in the d 35 samples from broilers not challenged with *Eimeria* spp. Forty taxa were specific for the challenged groups of which 24 appeared only in the NC group and 16 only in the SC group. Overall, *Eimeria* challenge had marginally significant effect (p = 0.06) on the total number of taxa in both the salinomycin and no-salinomycin group, but had significant effect (p = 0.007) on the composition of microbial communities and caused a change in the structures of the bacterial communities.

To determine the effect of salinomycin, *Eimeria* challenge and correlation with broiler performance parameters like age, body weight, coccidiosis lesion scores, hierarchical clustering and redundancy analysis (RDA) was performed. The model constructed with backward selection using all the available explanatory variables showed that salinomycin (p = 0.002) had significant effect and *Eimeria* challenge (p = 0.09) had marginally significant effect on cecal bacterial community structure. All other variables (age, body weight, *Eimeria* spp. lesion score) did not have significant impact on the distribution of bacterial communities and the sample distribution in the ordination space. The model explained 28.1% of the variation in the taxon abundance data. The RDA ordination plot (Fig. 3) displays groups of samples with similar taxon composition close together and the dissimilar samples far apart. The x-axis (RDA1) explained 20.4% and the y-axis (RDA2) explained 7.8%

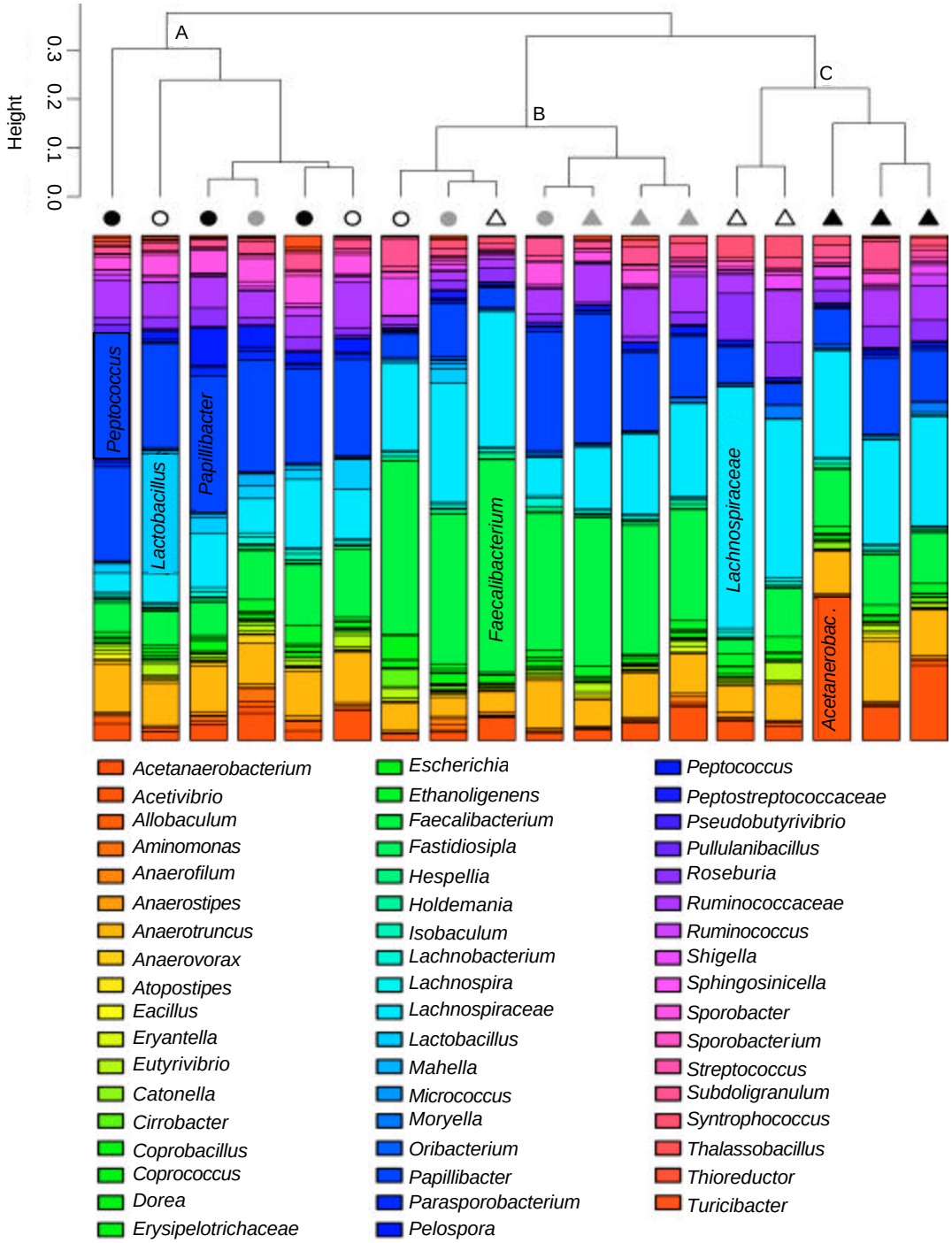

Fig. 2: Hierarchical clustering of the samples based on the cecal bacterial communities. Cecal contents DNA were examined from each of three chicks per treatment group. Circles represent no salinomycin in feed. Triangles represent samples from broilers supplemented with salinomycin. Blank symbols represent pre-challenge (d 28), black symbols post-challenge (d 35) and gray symbols represent post-challenge control (d 35) broilers. Group cluster labels were assigned as A = no salinomycin, B = post-challenge control, C = salinomycin. Bacterial abundance in each group was represented as stacked bar chart and is shown below the respective symbol

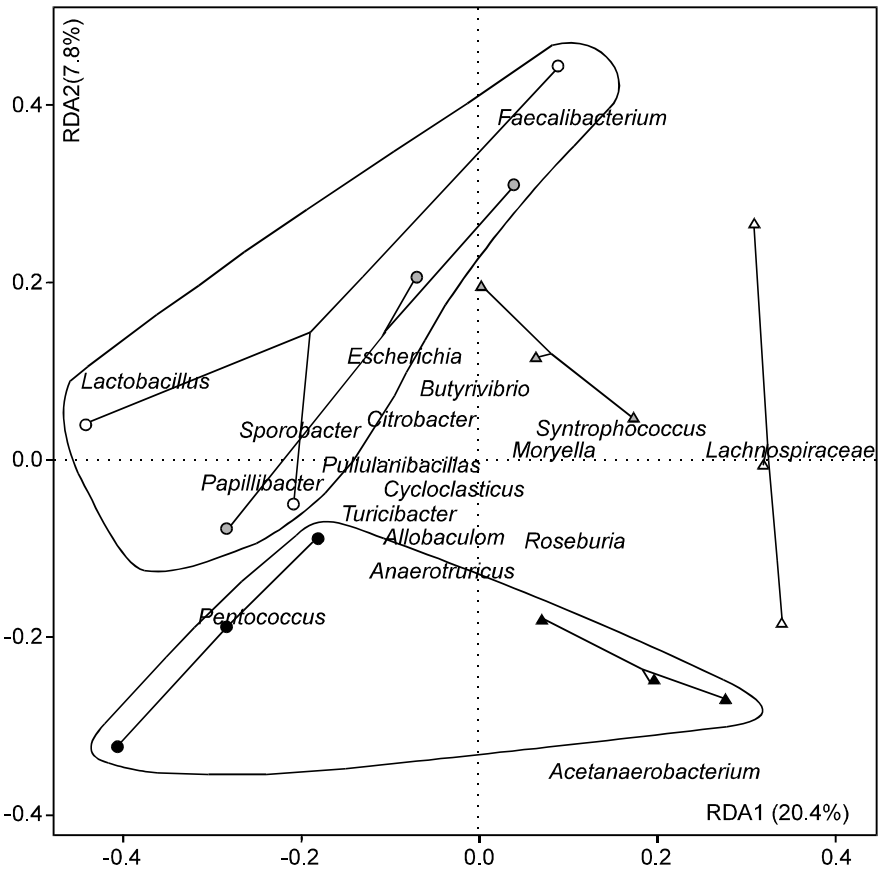

Fig. 3: Redundancy analysis showing the relationships between cecal bacterial communities from different treatment groups. Cecal contents DNA were examined from each of three chicks per treatment group. Circles represent no salinomycin in feed. Triangles represent samples from broilers supplemented with salinomycin. Blank symbols represent pre-challenge (d 28), black symbols post-challenge (d 35) and gray symbols represent post-challenge control (d 35) broilers. The solid outline (_) indicates non-challenged no salinomycin groups and the dashed outline (---) indicates *Eimeria*-challenged groups. Only the species that are significantly contributing to the spatial distribution of the samples in the ordination are shown. The model explained 28.1% of all the variability in the samples. The x-axis (RDA1) explained 20.4% of the taxon-salinomycin relationship (p = 0.002) and the second axis (RDA2) explained 7.8% of the taxon-*Eimeria* challenge relationship (p = 0.09)

of the variation in the taxon abundance in the samples. The x-axis corresponds to the salinomycin supplementation in the diet, separating the birds in the salinomycin groups (SN and SC, right side of the plot, p = 0.002) from birds which had no salinomycin (left side). The y-axis corresponds to the *Eimeria* challenge, separating the samples that were non-challenged (NN and SN) to the top of the x-axis and *Eimeria* spp. challenged samples (NC and SC, p = 0.09) to the bottom. The plot shows the clear separation of the SN group samples from the NN group. The post-challenged groups (d 35 NC and SC) were clearly separated from the non-challenged groups (NN and SN samples from d 28 and d 35).

The d 28 samples from the NN group were rich in *Lactobacillus* and *Faecalibacterium*, while the d 28 samples from the SN group were rich in *Lachnospiraceae*. The d 35 samples from SN and NN were present close to each on the ordination space. This proximity showed that these samples had similar bacterial community structure. The d 35 sample from NN was rich in *Papillibacter* and *Escherichia*, while the d 35 sample from SN was rich in *Syntrophococcus*. Completely different abundant taxa were observed in the d 35 samples in comparison of groups NC and SC. *Peptococcus* was rich in samples from the NC group, while *Acetanaerobacterium* was rich in samples from SC group. The species in the middle of the ordination space where commonly present in all the samples. These results from the RDA support the hierarchical clustering of samples using the taxon abundance data. The hierarchical clustering and multivariate redundancy

analysis provided statistical evidence and a concise assessment of the differences and similarities of bacterial microflora present in the ceca of broilers challenged with mixed *Eimeria* spp. and given diets supplemented with salinomycin.

This study is an addition to the research on molecular-based evaluation of intestinal microbial ecology of poultry livestock (Amit-Romach *et al*., 2004; Apajalahti *et al*., 2001, 2004; Callaway *et al*., 2009; Dowd *et al*., 2008a,b; Guo *et al*., 2004; Hume *et al*., 2006; Lu *et al*., 2003; Oviedo-Rondón *et al*., 2006a; Santos *et al*., 2007; Van der Wielen *et al*., 2002; Zhu *et al*., 2002). As reported earlier, the pyrosequencing-based studies have a few limitations (Acosta-Martinez *et al*., 2008; Callaway *et al*., 2009; Claesson *et al*., 2009; Dowd *et al*., 2008a,b; O' Day, 2008; Suchodolski *et al*., 2009) but these could be solved with the improvement of bacterial 16S rDNA database. Improving the methodology of relative abundance based analysis of pyrosequencing data would reduce the bias in the estimates of relative abundance data compared to the actual biological abundance (Amend *et al*., 2010). Overall this study has helped to further understand the diversity of cecal microbial communities, demonstrate a method to determine the dynamics of microbial communities and also establish the functionality of the microbial community with broiler performance.

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