Influence of dietary avilamycin on ileal and cecal microbiota in broiler chickens

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ABSTRACT The mechanisms by which antibiotic growth promoters (AGP) enhance growth rates, feed efficiencies, and disease resistance in poultry need to be understood for designing safer and alternative strategies to replace AGP. Avilamycin has been widely used as an AGP in poultry, but its impact on the structure and function of the gut microbiome of broiler chickens has not been fully elucidated. In this study, we investigated the bacterial communities of the ileum and cecum in broiler chickens fed with an avilamycin-supplemented diet, by high-throughput sequencing of bacterial 16S rRNA genes. Alpha diversity metrics indicated that the ileal bacterial diversity was higher in avilamycin-fed chickens than in the control group, whereas the opposite was true for the cecum. Multivariate analyses revealed that the ileal microbiota of the avilamycin-fed group were clearly distinguished from those of the control group, whereas the cecal bacterial communities were apparently not influenced by feeding diets containing avilamycin. In the ilea, 2 operational taxonomic units (OTU) that matched *Lactobacillus reuteri* and *Clostridium* were enriched ($P = 0.016$ and $P = 0.007$, respectively) in the avilamycin-fed group, and an OTU belonging to *Lactobacillus crispatus* was decreased ($P = 0.016$). In the cecal microbiota showing much higher diversity with 1,286 non-singleton OTU, 12 OTU were decreased, and 3 were increased in response to avilamycin treatment ($P = 0.005$–0.047). Functional profiling of bacterial communities based on PICRUSt analysis revealed that 10 functional categories were enriched by avilamycin treatments, and 4 functional categories were decreased. In conclusion, our results demonstrated that the influence of avilamycin supplementation on the diversity, taxonomic composition, and functional profiles of the microbiota was evidently different in the ileum and cecum. These results further our understanding of the impact of AGP on the composition and activity of commensal bacteria in the chicken gastrointestinal tract to develop novel feeding strategies for improving animal health and performance.

Key words: avilamycin, broiler chicken, microbiota, cecum, ileum

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INTRODUCTION

The chicken gut microbiota play important roles in digesting feeds, absorbing nutrients, synthesizing vitamins, and driving immune system development for host health and growth (Brisbin et al., 2008; Kohl, 2012; Yeoman et al., 2012). In poultry, various dietary components and feed additives have been used to modulate intestinal microbiota, as growth substrates for intestinal bacteria and as inhibitors of enteric pathogens (Engberg et al., 2004; Torok et al., 2011b). Therefore, it is important to understand the impact of feed additives on the composition and activity of commensal bacteria in the chicken gastrointestinal tract (**GIT**) to develop novel feeding strategies for improving animal health and performance. The chicken GIT has a complex architecture, with each section showing distinct microbial taxa and functions (Choi et al., 2014; Xiao et al., 2017). Recently, culture-independent approaches based on the 16S rRNA gene, including quantitative PCR, denaturing gradient gel electrophoresis (**DGGE**), terminal restriction fragment length polymorphism (**T-RFLP**), and high-throughput sequencing, have been employed to study the alterations brought about by antimicrobial feed additives in chicken GIT microbiota (Pedroso et al., 2006; Geier et al., 2009; Danzeisen et al., 2011).

Antibiotic growth promoters (**AGP**) have been widely used to improve growth performance and health of animals (Libby and Schaible, 1955; Dibner and Richards, 2005). Beneficial results from the use of AGP include reduced infectious disease burden, improved feed efficiency, and body weight gain (Jukes and Williams, 1953; Dafwang et al., 1984; Engster et al., 2002). Recent studies indicate that the function of AGP is more complex than merely affecting the enteric bacterial populations, directly or indirectly

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modulating host responses such as the immune system (Brown et al., 2017). Thus, a comprehensive understanding of the mechanisms by which the growth-promoting effects of AGP are induced remains to be fully resolved.

Avilamycin is an antibiotic of the orthomycin family known to inhibit the growth of Gram-positive bacteria by binding to and interfering with the function of ribosomes (Wolf, 1973; Kofoed and Vester, 2002) and have been widely used as AGP in poultry (Butaye et al., 2003; Castanon, 2007). Several studies have demonstrated that feeding avilamycin to chickens resulted in improved growth performance (Wellenreiter et al., 2000; Mountzouris et al., 2010). The enhanced growth performance of avilamycin-fed chickens is likely caused by improved nutrient digestibility, increased development of GIT structure, reduced anti-inflammatory responses, and decreased hematological signs of stress (Kim et al., 2011; La-ongkhum et al., 2011; Palamidi et al., 2016). Recently, studies based on high-throughput sequencing provided a detailed characterization of alterations in the cecal microbiota caused by dietary avilamycin treatment (Costa et al., 2017; Crisol-Martínez et al., 2017).

Previous studies of cecal and ileal microbiota of chickens suggested that the bacterial communities underwent rapid transitions in the early d of life (Torok et al., 2011a; Oakley et al., 2014a). The primary colonizers at the age of 1 to 3 d and 14 to 28 d were very different (Ballou et al., 2016). In the present study, we focused on the comparison of gut microbiota fully established after 35 d of growth under different dietary treatments rather than temporal changes during growth. To elucidate the bacterial taxa associated with avilamycin supplementation and their functions, we compared the ileal and cecal microbiota in 2 groups of broiler chickens fed with and without dietary avilamycin, by high-throughput sequencing of 16S rRNA gene amplicons. Comprehensive analyses of alterations in bacterial diversity, taxonomic composition, and predicted functional gene contents were performed for these purposes.

MATERIALS AND METHODS

Birds, Diets, and Sampling of Microbiota

A total of 100 one-day-old broiler chicks (ROSS 308) was obtained from a local hatchery (Yangji hatchery, Pyeongtaek, Republic of Korea) and raised in battery cages in an environmentally controlled room for 35 d, as described previously (Kim et al., 2014). Chicks were randomly allotted to one of 2 dietary treatments, and each treatment had 5 replicated cages with 10 birds per each cage. A commercial diet was prepared to meet or exceed energy and nutrient requirement for each growth phase of broiler chickens (NRC, 1994). Dietary treatment included the control-diet group with no avilamycin supplementation (**CD**) and avilamycin-diet group with 0.025 g/kg avilamycin supplementation (**AD**). Diets and water were provided ad libitum for the whole experiment. At the end of experiment, one bird with a body weight (**BW**) close to mean BW of each cage was euthanized by cervical dislocation, a method approved by the Animal Care Committee of Chung-Ang University. Intestinal contents from ileum and cecum regions of 5 randomly selected chickens were immediately collected and frozen at -80°C until analysis. The frozen samples were used for isolation of metagenomic DNA.

DNA Extration and Pyrosequencing of 16S rRNA Genes

Ileal and cecal contents from each sample were washed with 5 mL phosphate-buffered saline (**PBS**, pH 7.4) and centrifuged for 5 min at 13,000 rpm. The pellets from each sample were used for total genomic DNA extraction. DNA extraction was carried out using the QiaAmp DNA Stool Mini Kit (QIAGEN, Valencia, CA) according to the manufacturer's instructions. The V1-V3 region of the bacterial 16S rRNA gene was amplified by the fusion primers: V1–9F: 5′-X-AC-GAGTTTGATCMTGGCTCAG-3′ and V3–541R: 5′-X-AC-WTTACCGCGGCTGCTGG-3′, where X denotes a 7- to 11-nucleotide-long barcode uniquely designed for each DNA sample followed by a linker (AC). The 16S rRNA gene amplicons were generated through 25 cycles of PCR: 94°C for 30 s, 55°C for 45 s, and 72°C for 90 s, after initial denaturation at 94°C for 5 minutes. PCR was performed in triplicate, and amplicons from each sample were pooled and purified using the QIAquick PCR purification kit (QIAGEN). Equal amounts of amplicons from each sample were pooled and subjected to pyrosequencing on the Roche 454 Junior Sequencer (Roche, Brandford, CT) at Chunlab Inc. (Seoul, Korea).

Microbial Community Analyses

The raw reads were sorted into each sample according to sample barcodes, trimmed for the primer sequences, and quality-filtered by discarding reads with lengths <300 bp or the number of ambiguous bases >10, using the MOTHUR package (Schloss et al., 2009). Chimeric sequences were removed using UCHIME (Edgar et al., 2011). The pre-processed sequences were clustered to operational taxonomic units (**OTU**) at 3% distance, and the most abundant sequence type in each OTU was selected as the OTU-representative sequence. All singleton OTU were removed for further analyses. The taxonomic identity of OTU was inferred using the Ribosomal Database Project (**RDP**) training set version 14 (Cole et al., 2014) through a naïve Bayesian analysis method provided in the MOTHUR package. Bacterial species richness, diversity indices, and Good's coverage were estimated using 2,500 sequences subsampled from each sample, using MOTHUR. The OTU sequences assigned to the genus *Lactobacillus* were further classified at the species level. To achieve optimal

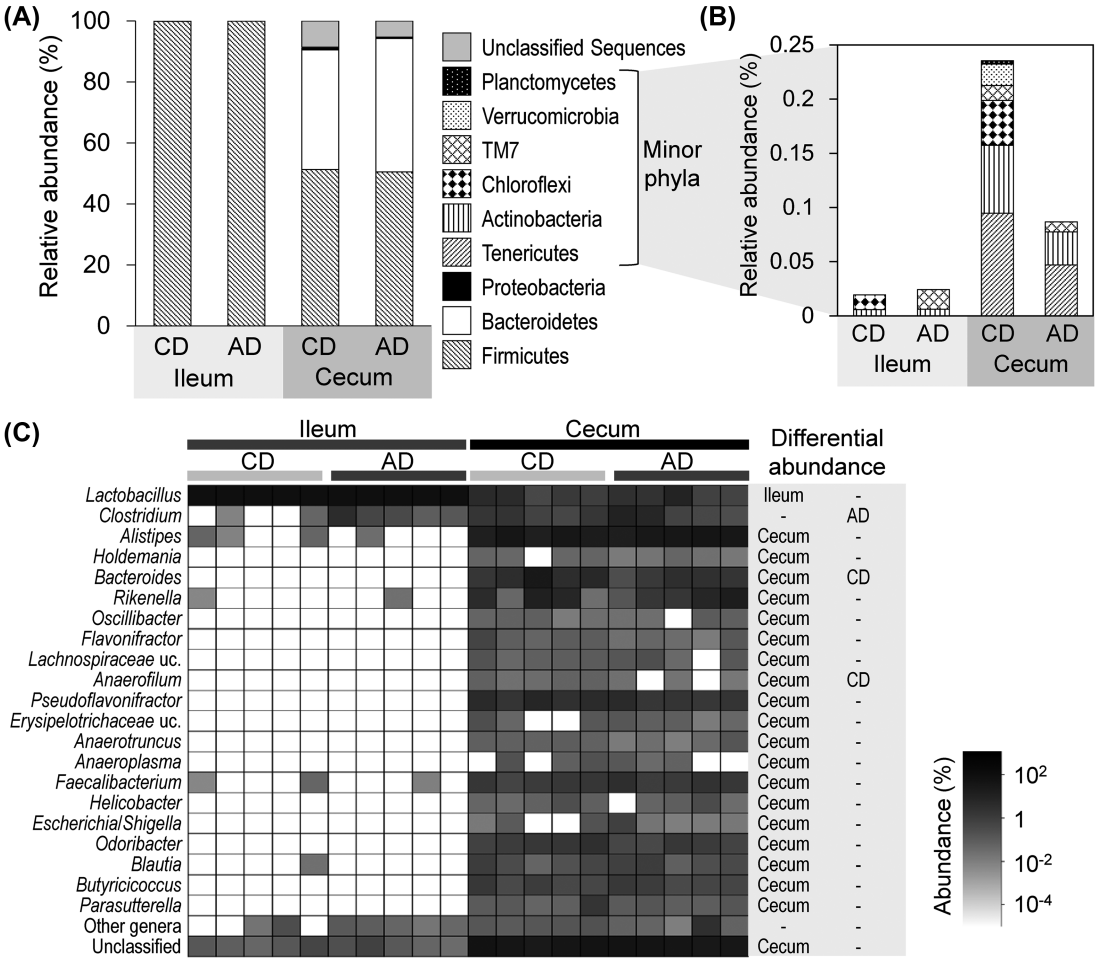

Figure 1. Bacterial community compositions of the ileum and cecum of avilamycin-supplemented diet (AD) and control diet (CD) group chickens. (A) Relative abundance of all bacterial phyla detected in the samples. The relative abundance values shown in the plot represent the averages of 5 samples in each treatment group. (B) Relative abundance of minor bacterial phyla. Six minor phyla that accounted for 0.1% or less of total microbiota are shown in the zoomed plot. (C) Heat map of relative abundance of bacterial genera detected in the samples. Bacterial genera that were significantly ($P < 0.05$) more abundant in ileal or cecal samples are marked by "Ileum" or "Cecum," respectively, on the right side of heat map. Bacterial genera that were significantly more abundant in one dietary treatment group than the other are also marked by "AD" or "CD," accordingly. Differential abundance was statistically tested using the linear discriminant analysis effect size (LEfSe) method.

classification at the species level, 16S rRNA sequences of the type strains of *Lactobacillus* spp. were obtained from the May 2015 release of RDP and used as a training set.

Analyses of Predicted Microbial Functions

Functional gene compositions of bacterial communities were predicted using the PICRUSt (phylogenetic investigation of communities by reconstruction of unobserved states) method (Langille et al., 2013). To generate BIOM-formatted files for PICRUSt input data, taxonomic classification was re-processed in MOTHUR with the GreenGenes V13.8 database (DeSantis et al., 2006). PICRUSt functionality was run using the MOTHUR-produced biom files via the Galaxy platform (Blankenberg et al., 2014; Afgan et al., 2016). Functional prediction was made using the KEGG orthologs database (Kanehisa et al., 2016) and summarized at the pathway hierarchy level 3.

Statistical Analyses

To compare the bacterial community structures across all samples, principal coordinate analysis (**PCoA**) was conducted. The Bray–Curtis dissimilarity index was used to construct a community dissimilarity matrix. The difference in community structures between the GIT sections and treatment groups were statistically tested by analysis of similarity (**ANOSIM**) (Clarke, 1993), non-parametric multivariate ANOVA (Adonis) (Anderson, 2001), and multiresponse permutation procedure (**MRPP**) (Zimmerman et al., 1985) with 999 permutations. Calculations of the Bray–Curtis matrix, PCoA, ANOSIM, Adonis, and MRPP were all performed using the Vegan package (Dixon, 2003). The alpha diversity indices of bacterial communities were compared between the AD and CD groups by independent 2-group t test implemented in R (Team, 2014). Statistical tests for differentially abundant OTU, genera, and functional categories were performed using the

linear discriminant analysis effect size (**LEfSe**) method (Segata et al., 2011) with an alpha value of 0.05 for the Kruskal–Wallis test among classes, and the threshold for the $\log_{10}$LDA score was set as 2.0. For analysis of differentially abundant functional categories, the quasi-likelihood F-test (**QLFT**) and likelihood ratio test (**LRT**) provided in the edgeR package (Robinson et al., 2010) and the Metastats method (White et al., 2009) also were applied in addition to the LEfSe method.

RESULTS AND DISCUSSION

Bacterial Community Structures of Ileal and Cecal Microbiota

Taxonomic compositions of the ileal and cecal bacterial communities were analyzed at the phylum and genus levels. All bacterial sequences from both intestinal regions were assigned to 9 phyla (Figure 1A). The ileal microbiota were dominated by the phylum *Firmicutes* (99.7 ± 0.2% of total reads), whereas the cecal microbiota were dominated by *Firmicutes* (49.7 ± 14.1%) and *Bacteroidetes* (42.1 ± 15.5%). Cecal samples shared *Proteobacteria* as a regular component (>0.1% in all samples) in addition to the 2 dominant phyla. The phyla *Actinobacteria, Chloroflexi, Plactomycetes, Tenericutes,* TM7, and *Verrucomicrobia* were occasionally included as minor members (average < 0.1%) (Figure 1B). Our results were consistent with those of previous studies, in that *Firmicutes, Bacteroidetes*, and *Proteobacteria* accounted for the majority of cecal bacteria (Choi et al., 2014; Mohd Shaufi et al., 2015; Costa et al., 2017). The abundance of *Bacteroidetes* in the cecum as estimated in our study (42.1% on average) was close to the result from Crisol-Martinez's "trial 2" chickens (44.2%) (Crisol-Martínez et al., 2017) and was higher than the values reported in other studies (Wei et al., 2013; Choi et al., 2014; Mohd Shaufi et al., 2015; Costa et al., 2017).

At the genus level, *Lactobacillus* was the only identifiable regular member of the ileal microbiota (99.0 ± 1.44%) and was more abundant in the ilea than in the ceca ($P < 0.001$). Cecal samples contained a number of core microbial taxa at the genus level, including *Alistipes, Bacteroides, Butyricicoccus, Faecalibacterium, Lactobacillus, Odoribacter*, and *Pseudoflavonibacter* with an abundance of >0.1% in all cecal samples (Figure 1C). The dominance of *Lactobacillus* observed in the ileal microbiota was consistent with previous suggestions that lactate fermentation by *Lactobacillus* is the main microbial function in the upper GIT (Stanley et al., 2014a; Oakley et al., 2014b; Stanley et al., 2014b). To our knowledge, there is little available information on the core microbial taxa of the chicken cecum. Our results indicate that the cecal microbiota share several genera as regular members, which may reflect the microbial functions in the chicken cecum. A complete list of abundance of all phyla and genera detected in this study are provided in Table S2 and Table S3.

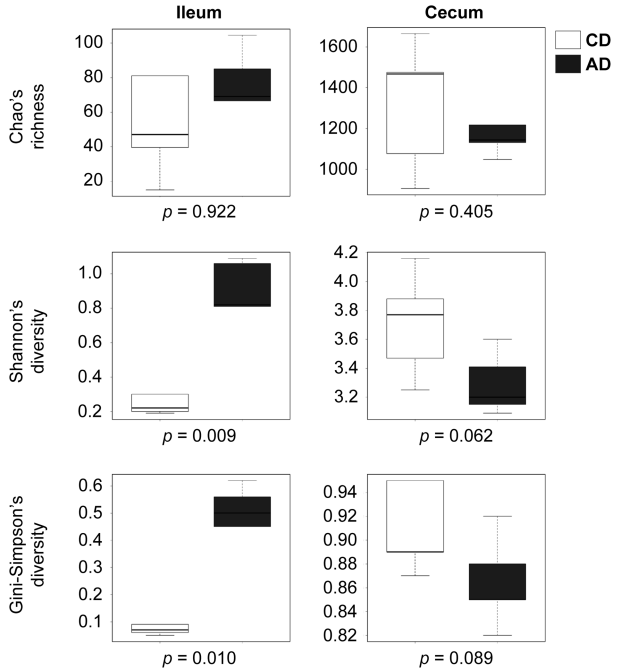

Figure 2. Shifts in bacterial alpha diversity by avilamycin treatment. Three different alpha diversity metrics, Chao's richness, Shannon's diversity, and Gini–Simpson's diversity indices, were compared between AD and CD groups. For all 3 indices, higher values correspond to greater diversity. *P*-values given below each boxplot were estimated by Student's *t* test.

Influence of Avilamycin on Diversity of Ileal and Cecal Microbiota

Based on the previous data on growth performance of chickens used in this experiment (Kim et al., 2014), the AD group displayed improved BW gain and feed conversion ratio (2,045 g and 1.52, respectively) compared to the CD group (1,941 g and 1.55, respectively). Similar improvements in BW gain and feed conversion ratio by feeding avilamycin-supplemented diets also were observed in previous experiments (Kim et al., 2011; Kaczmarek et al., 2016). We assumed that the distinctive properties of the ileal and cecal microbiota of the AD group could be associated with their improved growth performance. In this study, we present the results of how the ileal and cecal microbiota of the AD group differ from those of the CD group, based on the alpha- and beta-diversity statistics, taxonomic compositions, and predicted microbial functions.

Bacterial alpha diversity in the ileal and cecal microbiota of broiler chickens was estimated by calculating the number of OTU, and Chao's, Ace's, Shannon's, and Gini-Simpson's indices of richness and diversity (Table S3). The indices apparently indicated that cecal samples contained more diverse bacterial communities compared to ileal samples. Among ileal samples, the Chao's, Shannon's, and Gini-Simpson's indices were

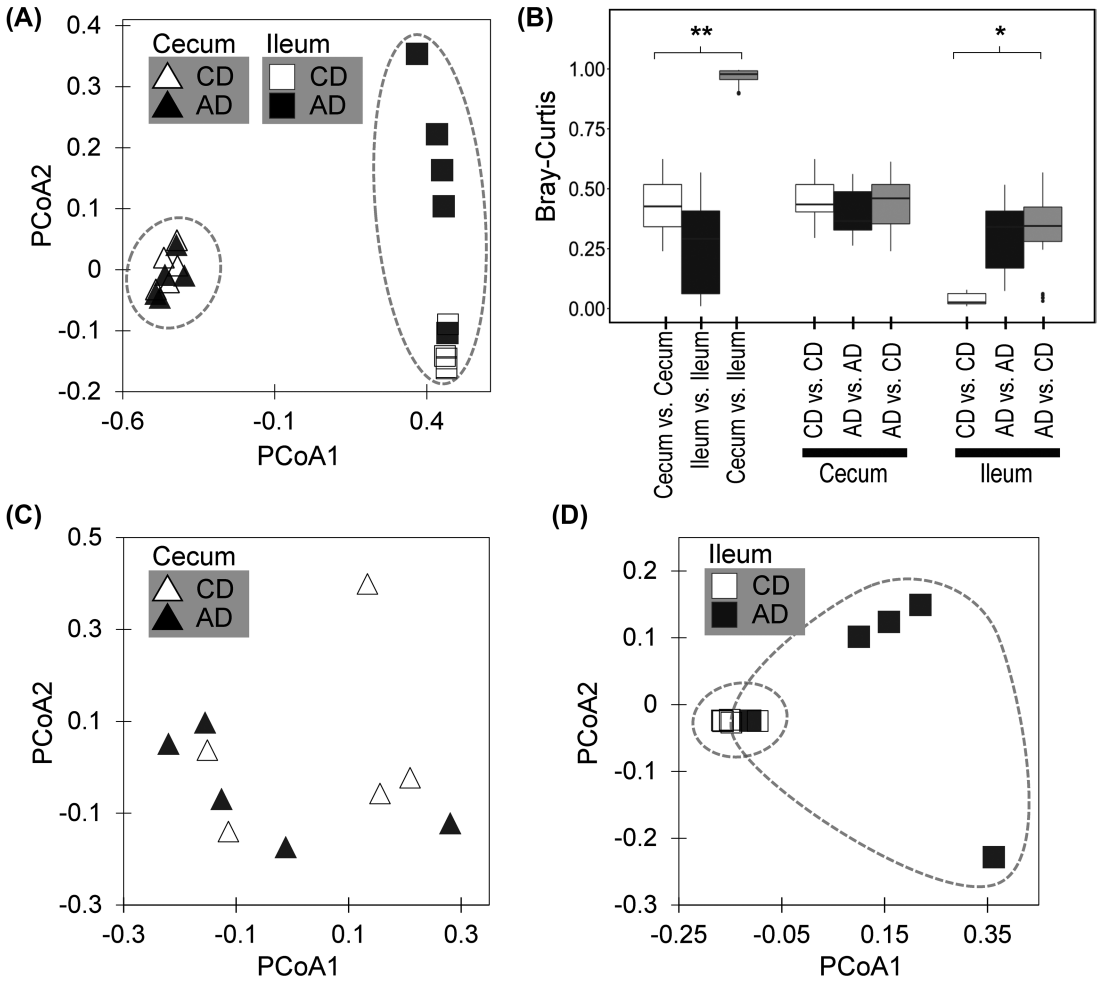

Figure 3. Influence of avilamycin treatment on bacterial beta diversity. (A) Principal coordinate analysis (PCoA) of all samples based on Bray–Curtis dissimilarity indices calculated from OTU abundance matrix. (B) Distribution of Bray–Curtis dissimilarity values from inter- and intra-group comparisons. Student's *t* test was performed for the differences between inter- and intra-group dissimilarities (*means $P < 0.05$ and **means $P < 0.01$). (C) PCoA plot for cecal samples. (D) PCoA plot for ileal samples.

higher in the AD group than in the CD group ($P = 0.922$, $P = 0.009$, and $P = 0.010$, respectively), suggesting that the overall bacterial diversity of ileal microbiota was increased by avilamycin treatment (Figure 2). In contrast, the AD group of cecal samples displayed lower average values of Chao's, Shannon's and Gini-Simpson's indices than the CD group ($P = 0.405$, $P = 0.062$, and $P = 0.089$, respectively), suggesting that avilamycin treatment resulted in decreased bacterial diversity of the cecal microbiota (Figure 2). Our results contrasted those of previous reports showing that avilamycin treatment had little impact on the alpha diversity of cecal bacteria (Costa et al., 2017; Crisol-Martínez et al., 2017). The influence of avilamycin on ileal bacterial diversity has not been examined in previous studies. According to our analysis, the effect of avilamycin treatment on bacterial alpha diversity was stronger in the ileum than in the cecum, with contrasting responses. Comparisons of the effects of avilamycin on bacterial diversity in the ileum and cecum have not been conducted in previous studies. In case of another AGP, virginiamycin treatment was found to have no significant effects on either ileal or cecal bacterial diversities (Pourabedin et al., 2015).

Beta-diversity analysis based on Bray–Curtis distances between the profiles of non-singleton OTU defined at a 97% similarity cutoff illustrated that the bacterial communities of the ileum and cecum were clearly distinguished (Figure 3A and 3B). The difference between the cecal and ileal bacterial community compositions was supported by the statistics obtained from ANOSIM ($R = 1$, $P = 0.001$), MRPP ($A = 0.49$, $P = 0.001$), and Adonis ($R^2 = 0.75$, $P = 0.001$) analyses. In the cecal microbiota, avilamycin treatment did not result in significant differentiation of bacterial community structures (Figure 3C; $R = 0.068$, $P = 0.218$ by ANOSIM; $A = 0.0094$, $P = 0.253$ by MRPP; and $R^2 = 0.13$, $P = 0.303$ by Adonis), and did not affect the overall beta diversity among the samples (Figure 3B). Among the ileal bacterial communities, the AD and CD samples displayed significantly different community compositions (Figure 3D; $R = 0.45$, $P = 0.022$ by ANOSIM; $A = 0.34$, $P = 0.014$ by MRPP; and $R^2 = 0.49$, $P = 0.023$ by Adonis), with

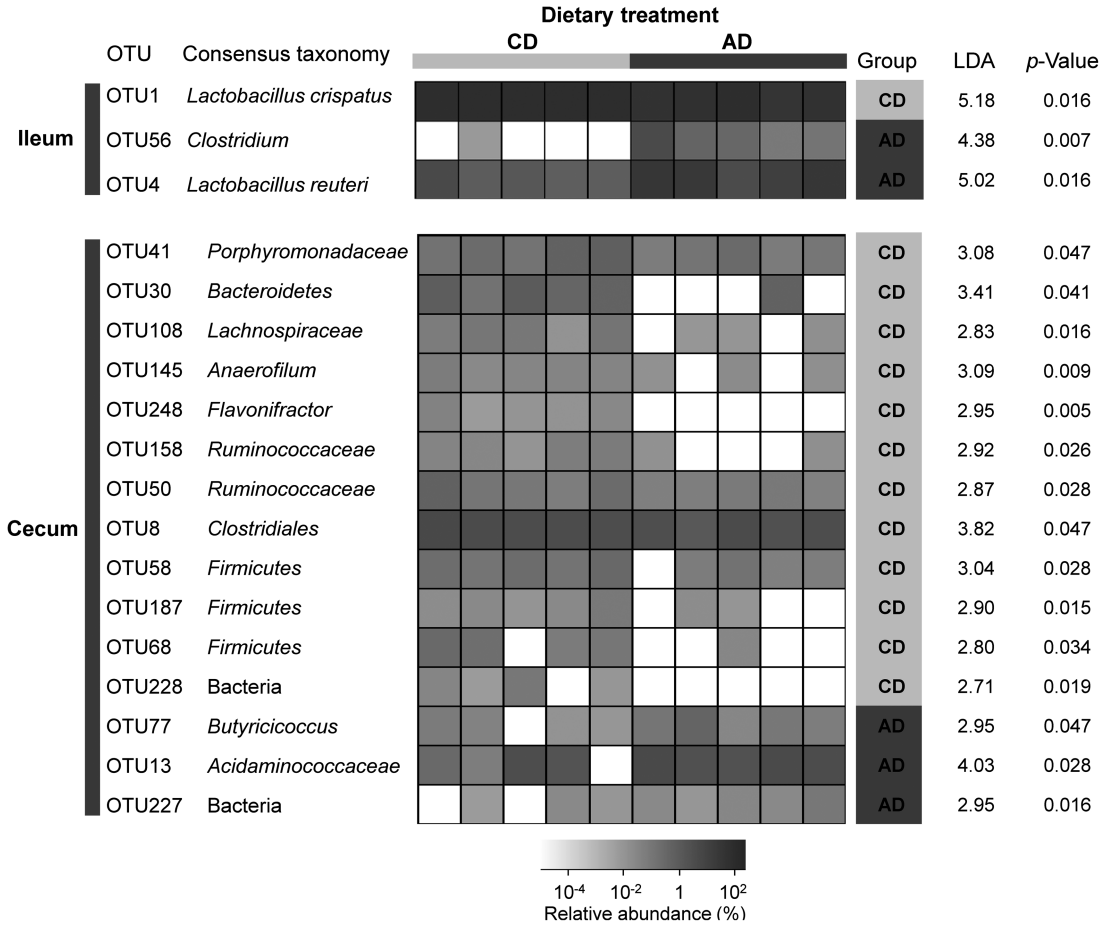

Figure 4. OTU whose abundance differed significantly between AD and CD groups. OTU were defined by the 97% similarity cut-off. Consensus taxonomic names of OTU were given at the lowest taxonomic level where each OTU was classified. Differential abundance was tested by linear discriminant analysis effect size (LEfSe) method. The class, log LDA score, and *P*-value determined by LEfSe test are displayed on the right side of the heat map.

increased beta diversity in AD samples (Figure 3B). The lack of statistically significant differences in the cecal bacterial community structures of AD and CD samples (Figure 3C) was in agreement with the previous studies (Costa et al., 2017; Crisol-Martínez et al., 2017). Unlike the cecal microbiota, the influence of avilamycin supplementation on bacterial community structure was evident in the ileal microbiota (Figure 3D), as proposed by earlier studies based on T-RFLP data (Torok et al., 2011a). The observed stability of the cecal microbiota might be due to its higher diversity resulting in stronger resilience. Resilience of a microbiome, which can be defined as the ability to return to an equilibrium state at the taxonomic or functional composition level following ecological stresses (Bäckhed et al., 2012; Lozupone et al., 2012), has been thought to be positively related with diversity (Shade et al., 2012).

Influence of Avilamycin on the Taxonomic Components of Ileal and Cecal Microbiota

To assess the differences induced by avilamycin supplementation in the bacterial community members of the ileal and cecal microbiota, we sorted the OTU whose abundance differed significantly between the AD and CD groups (Figure 4). Ileal samples were comprised of 74 non-singleton OTU, and 3 of these OTU showed significant differences between the AD and CD group samples. OTU4, which matched *Lactobacillus reuteri* with the highest similarity, and OTU56 belonging to *Clostridium* were enriched in the ileal samples of the AD group ($P = 0.016$ and $P = 0.007$, respectively). In contrast, OTU1, which showed the highest similarity with *Lactobacillus crispatus*, was decreased in the ilea of the AD group ($P = 0.016$). In cecal samples, 1,286 non-singleton OTU were identified, and of these, 12 OTU and 3 OTU were significantly decreased and increased, respectively, in response to avilamycin treatment. Three OTU that were increased in the AD cecal samples were classified as a species of *Butyricicoccus,* unknown *Acidaminococcaceae,* and unknown *Bacteria*, respectively. Among 12 OTU that decreased in the AD cecal samples, OTU248 belonging to *Flavonifractor* and OTU145 belonging to *Anaerofilum* exhibited the strongest *P*-values ($P = 0.005$ and $P = 0.009$, respectively). We also observed that the OTU of *Lachnospiraceae*, *Ruminococcaceae* and *Clostridiales* were less abundant in

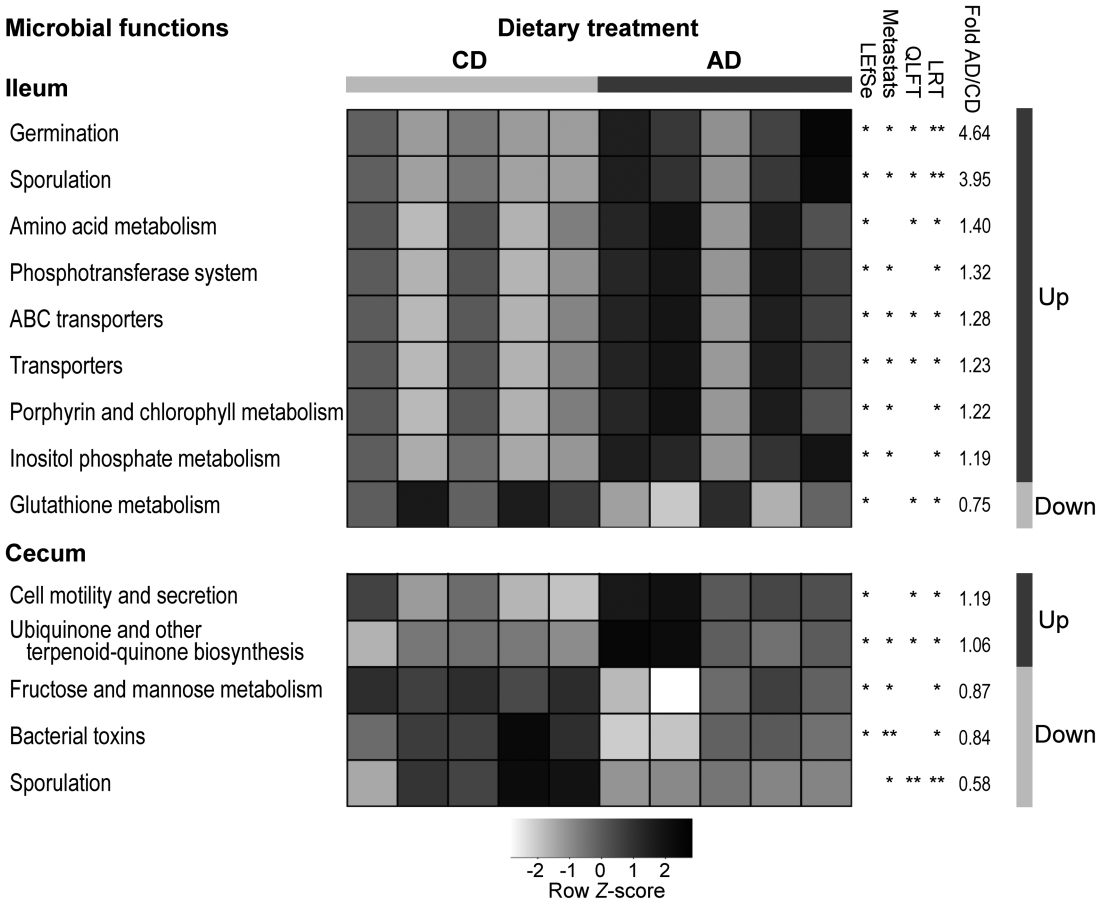

Figure 5. Predicted microbial functions enriched in the ilea and ceca of AD and CD group chickens. The heat map values were Z-score normalized from PICRUSt count values. Functional categories were taken from the KEGG pathway hierarchy level 3. Four different statistical tests were performed to evaluate the significance of differential abundance between AD and CD groups: linear discriminant analysis effect size (LEfSe), quasi-likelihood F-test (QLFT), likelihood ratio test (LRT), and the Metastats method. Significance levels determined from each test are displayed on the right side of heat map (*means $P < 0.05$ and **means $P < 0.01$). Average fold difference between AD and CD groups are provided. Functional categories that increased in the AD group are shaded by gray color.

the AD ceca, which is consistent with the results of the previous study based on T-RFLP (Torok et al., 2011a).

Specific attention should be given to the identity of OTU that were enriched by avilamycin treatment, as these taxa could represent candidate probiotics for alternative feed additives for AGP. In the cecum, previous studies have shown OTU of *Subdoligranulum* (Torok et al., 2011a) and *Suterellaceae* (Costa et al., 2017) to be positively associated with avilamycin treatment. In contrast, our study showed *Butyricicoccus* and *Acidaminococcaceae* OTU to be associated with avilamycin treatment. The observed enrichment of a *Butirycicoccus* OTU in the ceca of the AD group is interesting because a previous study showed that dietary administration of a *Butyricicoccus* isolate as a probiotic to broiler chickens resulted in improved feed efficiency and increased resistance to necrotic enteritis caused by *C. perfringens* (Eeckhaut et al., 2016). *Butyricicoccus* is a butyrate producer and short chain fatty acids (**SCFA**) are known to promote the growth of enterocytes, lower pH, and inhibit the colonization of some pathogens (van der Wielen et al., 2000).

Impact of Avilamycin on the Predicted Functions of Ileal and Cecal Microbiota

Functional profiling of bacterial communities was predicted using PICRUSt on the basis of the closest reference genomes that matched the OTU found in the 16S rRNA sequence data (Langille et al., 2013). The predicted gene contents were converted to the abundance matrix of KEGG functional categories. Despite some limitations in the prediction, inspection of functional categories likely enriched or depleted in the given bacterial community may provide insights into the microbiome-mediated pathways involved in the growth-promoting effects of avilamycin. We found that 8 and 2 functional categories were enriched by avilamycin treatment in the ileal and cecal bacterial communities, respectively, and one and 3 functional categories were

Table 1. Summary on the characteristics of ileal and cecal microbial communities of avilamycin-fed chickens.

	Ileum	Cecum
Bacterial alpha diversity[1]	AD > CD	AD < CD
Bacterial beta diversity[2]	AD > CD	Not changed
Significant changes in microbiota between of AD and CD groups[3]	Yes	No
Taxonomy of enriched OTU[4]	*Lactobacillus reuteri Clostridium* (N = 2)	*Butyricicoccus Acidaminococcaceae* Unclassified bacteria (N = 3)
Taxonomy of reduced OTU[4]	*Lactobacillus crispatus* (N = 1)	*Flavonifractor*, *Anaerofilum*, *Lachnospiraceae*, Bacteroidetes, *Ruminococcaceae*, *Clostridiales*, Firmicutes, Unclassified bacteria (N = 12)

[1]Bacterial alpha diversity was compared between control-diet (CD) and avilamycin-diet (AD) groups based on Shannon's and Gini–Simpson's diversity indices.
[2]Bacterial beta diversity was compared between CD and AD groups based on Bray–Curtis dissimilarity values.
[3]Changes in microbiota were evaluated by ANOSIM, MRPP, Adonis, and PCoA analyses.
[4]LEfSe analysis was used to select the OTU enriched or reduced in the AD group.

decreased in the ileal and cecal microbiota, respectively, considering only the functional categories of bacterial orthologs whose abundances were significantly different ($P < 0.05$) by at least 3 of the 4 statistical tests (Figure 5). Many of the predicted functions enriched in the ileum and cecum of the AD group have been reportedly associated with host physiology in studies on human and animal gut microbiomes. Enrichment of the phosphotransferase system and ABC transporters in the gut microbiome have been associated with the altered status of host diets or energy metabolism (Turnbaugh et al., 2009; Everard et al., 2014; Kreznar et al., 2017). Amino acid metabolism has been observed to be enriched in the GIT of mice and humans on a high-fat diet (Yatsunenko et al., 2012; Daniel et al., 2014) and related to the downstream SCFA production (Smith and Mcfarlane, 1997; Louis and Flint, 2017). Sporulation and germination, inositol phosphate metabolism, porphyrin and chlorophyll metabolism, cell motility, and ubiquinone biosynthesis also are reported to be associated with dietary lifestyle or obesity in human and animal studies (Leone et al., 2015; Guo et al., 2016; Rajpal et al., 2016).

CONCLUSION

In the present study, the cecal and ileal bacterial communities of broiler chickens fed with and without dietary avilamycin were analyzed in relation to the improved growth performance of the avilamycin-fed group. Our results demonstrated that ileal and cecal microbiomes differed considerably in terms of community diversity metrics in response to dietary avilamycin treatment; ileal microbiota were influenced more significantly, whereas cecal microbiota remained relatively stable. In addition, we found a number of key bacterial taxa and functions that characterized the ileum and cecum of avilamycin-fed chickens. Influence of avilamycin on the ileal and cecal microbiota observed in this study is summarized in Table 1. These results may provide useful information for developing alternative feed additives to AGP to improve health and production of chickens.

SUPPLEMENTARY DATA

Supplementary data are available at *Poultry Science* online.

Table S1. Relative abundance of bacterial phyla in each sample.

Table S2. Relative abundance of bacterial genera in each sample.

Table S3. Bacterial community alpha diversity indices of the samples.

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REFERENCES

Afgan, E., D. Baker, M., Beek, D. Blankenberg, D. Bouvier, M. Čech, J. Chilton, D. Clements, N. Coraor, C. Eberhard, B. Grüning, A. Guerler, J. Hillman-Jackson, G. Von Kuster, E. Rasche, N. Soranzo, N. Turaga, J. Taylor, A. Nekrutenko, and J. Goecks. 2016. The Galaxy platform for accessible, reproducible and collaborative biomedical analyses: 2016 update. Nucleic Acids Res. 44:W3–W10.

Anderson, M. J. 2001. A new method for non-parametric multivariate analysis of variance. Austral Ecol. 26:32–46.

Bäckhed, F., C. M. Fraser, Y. Ringel, M. E. Sanders, R. B. Sartor, P. M. Sherman, J. Versalovic, V. Young, and B. B. Finlay. 2012. Defining a healthy human gut microbiome: current concepts, future directions, and clinical applications. Cell Host & Microbe. 12:611–622.

Ballou, A. L., R. A. Ali, M. A. Mendoza, J. C. Ellis, H. M. Hassan, W. J. Croom, and M. D. Koci. 2016. Development of the chick microbiome: How early exposure influences future microbial diversity. Front. Vet. Sci. 3:2.

Blankenberg, D., G. Von Kuster, E. Bouvier, D. Baker, E. Afgan, N. Stoler, J. Taylor, and A. Nekrutenko. 2014. Dissemination of scientific software with Galaxy ToolShed. Genome Biol. 15:403.

Brisbin, J. T., J. Gong, and S. Sharif. 2008. Interactions between commensal bacteria and the gut-associated immune system of the chicken. Anim. Health Res. Rev. 9:101–110.

Brown, K., R. R. E. Uwiera, M. L. Kalmokoff, S. P. J. Brooks, and G. D. Inglis. 2017. Antimicrobial growth promoter use in livestock: a requirement to understand their modes of action to develop effective alternatives. Int. J. Antimicrob. Agents. 49:12–24.

Butaye, P., L. A. Devriese, and F. Haesebrouck. 2003. Antimicrobial growth promoters used in animal feed: Effects of less well

known antibiotics on gram-positive bacteria. Clin. Microbiol. Rev. 16:175–188.
Castanon, J. I. R. 2007. History of the use of antibiotic as growth promoters in European poultry feeds. Poult. Sci. 86:2466–2471.
Choi, J. H., G. B. Kim, and C. J. Cha. 2014. Spatial heterogeneity and stability of bacterial community in the gastrointestinal tracts of broiler chickens. Poult. Sci. 93:1942–1950.
Clarke, K. R. 1993. Non-parametric multivariate analyses of changes in community structure. Aust. J. Ecol. 18:117–143.
Cole, J. R., Q. Wang, J. A. Fish, B. Chai, D. M. McGarrell, Y. Sun, C. T. Brown, A. Porras-Alfaro, C. R. Kuske, and J. M. Tiedje. 2014. Ribosomal Database Project: Data and tools for high throughput rRNA analysis. Nucleic Acids Res. 42:D633–D642.
Costa, M. C., J. A. Bessegatto, A. A. Alfieri, J. S. Weese, J. A. B. Filho, and A. Oba. 2017. Different antibiotic growth promoters induce specific changes in the cecal microbiota membership of broiler chicken. PLoS One. 12:e0171642.
Crisol-Martínez, E., D. Stanley, M. S. Geier, R. J. Hughes, and R. J. Moore. 2017. Understanding the mechanisms of zinc bacitracin and avilamycin on animal production: Linking gut microbiota and growth performance in chickens. Appl. Microbiol. Biotechnol. 101:4547–4559.
Dafwang, I. I., H. R. Bird, and M. L. Sunde. 1984. Broiler chick growth response to antibiotics, 1981–19821. Poult. Sci. 63:1027–1032.
Daniel, H., A. M. Gholami, D. Berry, C. Desmarchelier, H. Hahne, G. Loh, S. Mondot, P. Lepage, M. Rothballer, A. Walker, C. Bohm, M. Wenning, M. Wagner, M. Blaut, P. Schmitt-Kopplin, B. Kuster, D. Haller, and T. Clavel. 2014. High-fat diet alters gut microbiota physiology in mice. ISME J. 8:295–308.
Danzeisen, J. L., H. B. Kim, R. E. Isaacson, Z. J. Tu, and T. J. Johnson. 2011. Modulations of the chicken cecal microbiome and metagenome in response to anticoccidial and growth promoter treatment. PLoS One. 6:e27949.
DeSantis, T. Z., P. Hugenholtz, N. Larsen, M. Rojas, E. L. Brodie, K. Keller, T. Huber, D. Dalevi, P. Hu, and G. L. Andersen. 2006. Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB. Appl. Environ. Microbiol. 72:5069–5072.
Dibner, J. J., and J. D. Richards. 2005. Antibiotic growth promoters in agriculture: history and mode of action. Poult. Sci. 84:634–643.
Dixon, P. 2003. VEGAN, a package of R functions for community ecology. J. Veg. Sci. 14:927–930.
Edgar, R. C., B. J. Haas, J. C. Clemente, C. Quince, and R. Knight. 2011. UCHIME improves sensitivity and speed of chimera detection. Bioinformatics. 27:2194–2200.
Eeckhaut, V., J. Wang, A. Van Parys, F. Haesebrouck, M. Joossens, G. Falony, J. Raes, R. Ducatelle, and F. Van Immerseel. 2016. The probiotic *Butyricicoccus pullicaecorum* reduces feed conversion and protects from potentially harmful intestinal microorganisms and necrotic enteritis in broilers. Front. Microbiol. 7:1416.
Engberg, R. M., M. S. Hedemann, S. Steenfeldt, and B. B. Jensen. 2004. Influence of whole wheat and xylanase on broiler performance and microbial composition and activity in the digestive tract. Poult. Sci. 83:925–938.
Engster, H. M., D. Marvil, and B. Stewart-Brown. 2002. The effect of withdrawing growth promoting antibiotics from broiler chickens: A long-term commercial industry study. J. Appl. Poult. Res. 11:431–436.
Everard, A., V. Lazarevic, N. Gaia, M. Johansson, M. Stahlman, F. Backhed, N. M. Delzenne, J. Schrenzel, P. Francois, and P. D. Cani. 2014. Microbiome of prebiotic-treated mice reveals novel targets involved in host response during obesity. ISME J. 8:2116–2130.
Geier, M. S., V. A. Torok, G. E. Allison, K. Ophel-Keller, and R. J. Hughes. 2009. Indigestible carbohydrates alter the intestinal microbiota but do not influence the performance of broiler chickens. J. Appl. Microbiol. 106:1540–1548.
Guo, Z., J. Zhang, Z. Wang, K. Y. Ang, S. Huang, Q. Hou, X. Su, J. Qiao, Y. Zheng, L. Wang, E. Koh, H. Danliang, J. Xu, Y. K. Lee, and H. Zhang. 2016. Intestinal microbiota distinguish gout patients from healthy humans. Sci. Rep. 6:20602.
Jukes, T. H., and W. L. Williams. 1953. Nutritional effects of antibiotics. Pharmacol. Rev. 5:381–420.
Kaczmarek, S. A., A. Barri, M. Hejdysz, and A. Rutkowski. 2016. Effect of different doses of coated butyric acid on growth performance and energy utilization in broilers. Poult. Sci. 95:851–859.
Kanehisa, M., Y. Sato, M. Kawashima, M. Furumichi, and M. Tanabe. 2016. KEGG as a reference resource for gene and protein annotation. Nucleic Acids Res. 44:D457–D462.
Kim, G. B., Y. M. Seo, C. H. Kim, and I. K. Paik. 2011. Effect of dietary prebiotic supplementation on the performance, intestinal microflora, and immune response of broilers. Poult. Sci. 90:75–82.
Kim, J. H., J. W. Kim, B. B. Lee, G. I. Lee, J. H. Lee, G. B. Kim, and D. Y. Kil. 2014. Effect of dietary supplementation of bacteriophage on growth performance and cecal bacterial populations in broiler chickens raised in different housing systems. Livest. Sci. 170:137–141.
Kofoed, C. B., and B. Vester. 2002. Interaction of avilamycin with ribosomes and resistance caused by mutations in 23S rRNA. Antimicrob. Agents Chemother. 46:3339–3342.
Kohl, K. 2012. Diversity and function of the avian gut microbiota. J. Comp. Physiol. B. 182:591–602.
Kreznar, J. H., M. P. Keller, L. L. Traeger, M. E. Rabaglia, K. L. Schueler, D. S. Stapleton, W. Zhao, E. I. Vivas, B. S. Yandell, A. T. Broman, B. Hagenbuch, A. D. Attie, and F. E. Rey. 2017. Host genotype and gut microbiome modulate insulin secretion and diet-induced metabolic phenotypes. Cell Rep. 18:1739–1750.
La-ongkhum, O., N. Pungsungvorn, N. Amornthewaphat, and S. Nitisinprasert. 2011. Effect of the antibiotic avilamycin on the structure of the microbial community in the jejunal intestinal tract of broiler chickens. Poult. Sci. 90:1532–1538.
Langille, M. G. I., J. Zaneveld, J. G. Caporaso, D. McDonald, D. Knights, J. A. Reyes, J. C. Clemente, D. E. Burkepile, R. L. Vega Thurber, R. Knight, R. G. Beiko, and C. Huttenhower. 2013. Predictive functional profiling of microbial communities using 16S rRNA marker gene sequences. Nat. Biotechnol. 31:814–821.
Leone, V., Sean, M. Gibbons, K. Martinez, Alan, L. Hutchison, Edmond, Y. Huang, Candace, M. Cham, Joseph, F. Pierre, Aaron, F. Heneghan, A. Nadimpalli, N. Hubert, E. Zale, Y. Wang, Y. Huang, B. Theriault, Aaron, R. Dinner, Mark, W. Musch, Kenneth, A. Kudsk, Brian, J. Prendergast, Jack, A. Gilbert, Eugene, and B. Chang. 2015. Effects of diurnal variation of gut microbes and high-fat feeding on host circadian clock function and metabolism. Cell Host & Microbe. 17:681–689.
Libby, D. A., and P. J. Schaible. 1955. Observations on growth responses to antibiotics and arsonic acids in poultry feeds. Science. 121:733–734.
Louis, P., and H. J. Flint. 2017. Formation of propionate and butyrate by the human colonic microbiota. Environ. Microbiol. 19:29–41.
Lozupone, C. A., J. I. Stombaugh, J. I. Gordon, J. K. Jansson, and R. Knight. 2012. Diversity, stability and resilience of the human gut microbiota. Nature. 489:220–230.
Mohd Shaufi, M. A., C. C. Sieo, C. W. Chong, H. M. Gan, and Y. W. Ho. 2015. Deciphering chicken gut microbial dynamics based on high-throughput 16S rRNA metagenomics analyses. Gut Pathog. 7:4.
Mountzouris, K. C., P. Tsitrsikos, I. Palamidi, A. Arvaniti, M. Mohnl, G. Schatzmayr, and K. Fegeros. 2010. Effects of probiotic inclusion levels in broiler nutrition on growth performance, nutrient digestibility, plasma immunoglobulins, and cecal microflora composition. Poult. Sci. 89:58–67.
NRC. 1994. Nutrient Requirements of Poultry. 9th rev. ed. Natl. Acad. Press, Washington, DC.
Oakley, B. B., R. J. Buhr, C. W. Ritz, B. H. Kiepper, M. E. Berrang, B. S. Seal, and N. A. Cox. 2014a. Successional changes in the chicken cecal microbiome during 42 days of growth are independent of organic acid feed additives. BMC Vet. Res. 10:282.
Oakley, B. B., H. S. Lillehoj, M. H. Kogut, W. K. Kim, J. J. Maurer, A. Pedroso, M. D. Lee, S. R. Collett, T. J. Johnson, and N. A. Cox. 2014b. The chicken gastrointestinal microbiome. FEMS Microbiol. Lett. 360:100–112.
Palamidi, I., K. Fegeros, M. Mohnl, W. H. A. Abdelrahman, G. Schatzmayr, G. Theodoropoulos, and K. C. Mountzouris. 2016.

Probiotic form effects on growth performance, digestive function, and immune related biomarkers in broilers. Poult. Sci. 95:1598–1608.

Pedroso, A. A., J. F. Menten, M. R. Lambais, A. M. C. Racanicci, F. A. Longo, and J. O. B. Sorbara. 2006. Intestinal bacterial community and growth performance of chickens fed diets containing antibiotics. Poult. Sci. 85:747–752.

Pourabedin, M., L. Guan, and X. Zhao. 2015. Xylo-oligosaccharides and virginiamycin differentially modulate gut microbial composition in chickens. Microbiome. 3:15.

Rajpal, D. K., J.-L. Klein, D. Mayhew, J. Boucheron, A. T. Spivak, V. Kumar, K. Ingraham, M. Paulik, L. Chen, S. Van Horn, E. Thomas, G. Sathe, G. P. Livi, D. J. Holmes, and J. R. Brown. 2016. Selective spectrum antibiotic modulation of the gut microbiome in obesity and diabetes rodent models. PLoS One. 10:e0145499.

Robinson, M. D., D. J. McCarthy, and G. K. Smyth. 2010. edgeR: A Bioconductor package for differential expression analysis of digital gene expression data. Bioinformatics. 26:139–140.

Schloss, P. D., S. L. Westcott, T. Ryabin, J. R. Hall, M. Hartmann, E. B. Hollister, R. A. Lesniewski, B. B. Oakley, D. H. Parks, C. J. Robinson, J. W. Sahl, B. Stres, G. G. Thallinger, D. J. Van Horn, and C. F. Weber. 2009. Introducing mothur: Open-source, platform-independent, community-supported software for describing and comparing microbial communities. Appl. Environ. Microbiol. 75:7537–7541.

Segata, N., J. Izard, L. Waldron, D. Gevers, L. Miropolsky, W. S. Garrett, and C. Huttenhower. 2011. Metagenomic biomarker discovery and explanation. Genome Biol. 12:R60.

Shade, A., H. Peter, S. Allison, D. Baho, M. Berga, H. Buergmann, D. Huber, S. Langenheder, J. Lennon, J. Martiny, K. Matulich, T. Schmidt, and J. Handelsman. 2012. Fundamentals of microbial community resistance and resilience. Front. Microbiol. 3:417.

Smith, E. A., and G. T. Macfarlane. 1997. Dissimilatory amino acid metabolism in human colonic bacteria. Anaerobe. 3:327–337.

Stanley, D., M. S. Geier, R. J. Hughes, S. E. Denman, and R. J. Moore. 2014a. Highly variable microbiota development in the chicken gastrointestinal tract. PLoS One. 8:e84290.

Stanley, D., R. J. Hughes, and R. J. Moore. 2014b. Microbiota of the chicken gastrointestinal tract: influence on health, productivity and disease. Appl. Microbiol. Biotechnol. 98:4301–4310.

Team, R. C. 2014. R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria.

Torok, V. A., G. E. Allison, N. J. Percy, K. Ophel-Keller, and R. J. Hughes. 2011a. Influence of antimicrobial feed additives on broiler commensal posthatch gut microbiota development and performance. Appl. Environ. Microbiol. 77:3380–3390.

Torok, V. A., R. J. Hughes, L. L. Mikkelsen, R. Perez-Maldonado, K. Balding, R. MacAlpine, N. J. Percy, and K. Ophel-Keller. 2011b. Identification and characterization of potential performance-related gut microbiotas in broiler chickens across various feeding trials. Appl. Environ. Microbiol. 77:5868–5878.

Turnbaugh, P. J., V. K. Ridaura, J. J. Faith, F. E. Rey, R. Knight, and J. I. Gordon. 2009. The effect of diet on the human gut microbiome: A metagenomic analysis in humanized gnotobiotic mice. Sci. Transl. Med. 1:6ra14–16ra14.

van der Wielen, P. W. J. J., S. Biesterveld, S. Notermans, H. Hofstra, B. A. P. Urlings, and F. van Knapen. 2000. Role of volatile fatty acids in development of the cecal microflora in broiler chickens during growth. Appl. Environ. Microbiol. 66:2536–2540.

Wei, S., M. Morrison, and Z. Yu. 2013. Bacterial census of poultry intestinal microbiome. Poult. Sci. 92:671–683.

Wellenreiter, R. H., D. H. Mowrey, L. A. Stobbs, and J. A. d'Assonville. 2000. Effects of avilamycin on performance of broiler chickens. Vet. Ther. 1:118–124.

White, J. R., N. Nagarajan, and M. Pop. 2009. Statistical methods for detecting differentially abundant features in clinical metagenomic samples. PLoS Comp. Biol. 5:e1000352.

Wolf, H. 1973. Avilamycin, an inhibitor of the 30S ribosomal subunits function. FEBS Lett. 36:181–186.

Xiao, Y., Y. Xiang, W. Zhou, J. Chen, K. Li, and H. Yang. 2017. Microbial community mapping in intestinal tract of broiler chicken. Poult. Sci. 96:1387–1393.

Yatsunenko, T., F. E. Rey, M. J. Manary, I. Trehan, M. G. Dominguez-Bello, M. Contreras, M. Magris, G. Hidalgo, R. N. Baldassano, A. P. Anokhin, A. C. Heath, B. Warner, J. Reeder, J. Kuczynski, J. G. Caporaso, C. A. Lozupone, C. Lauber, J. C. Clemente, D. Knights, R. Knight, and J. I. Gordon. 2012. Human gut microbiome viewed across age and geography. Nature. 486:222–227.

Yeoman, C. J., N. Chia, P. Jeraldo, M. Sipos, N. D. Goldenfeld, and B. A. White. 2012. The microbiome of the chicken gastrointestinal tract. Anim. Health Res. Rev. 13:89–99.

Zimmerman, G. M., H. Goetz, and P. W. Mielke. 1985. Use of an improved statistical method for group comparisons to study effects of prairie fire. Ecology. 66:606–611.