**INTRODUCTION**

Antibiotic growth promoters (AGP) are administered in a low, subtherapeutic dosage to inhibit infections from pathogenic bacteria as well as to allow better digestion of food in animals, thereby promoting muscle growth in the animals (Hughes and Heritage, 2002). There is a significant increase in BW of chickens with inclusion of AGP in the poultry feed (Bunyan et al., 1977), especially the inclusion of penicillin, which has been linked to increased growth performance in broilers (Diarra et al., 2007). Administration of AGP is considered to cause significant loss in weight of the small intestine and thinning of the gut wall, thereby increasing the absorption of nutrients (Coates, et al., 1955; Madge, 1969) as well as reducing competition for nutrients and sparing them for the host (Dibner and Richards, 2005). Hence, AGP may have a direct effect on the gut microbiota and the increase in BW in the chickens may also be related to alteration of the gut microbial composition.

Studies in mice, pigs, and humans have shown a similar shift in gut microbiota in obese individuals compared with the lean ones, indicating that this microbial shift could be responsible for the increase in energy harvest and BW. The results of this study suggest that the growth-promoting effect of penicillin supplementation in broilers may be mediated by a similar microbial process.

**Key words:** chicken, antibiotics growth promoter, 16S rRNA gene, cecal microbiota
this overall change related to diet and increased energy harvest in broiler chickens (Muramatsu et al., 1994; Torok et al., 2008).

We aim at understanding this alteration in gut microbiota of broiler chickens that have been fed diet with (ANT) and without (CON) penicillin, where a significant difference in BW of the chickens was noted between the 2 groups (Karimi et al., 2010). Community DNA was pooled from 5 broilers from each of the 2 groups, which has been shown as an appropriate number that can lead to accurate estimation of AGP effect on gut microbiota (Zhou et al., 2007). For this, we make the use of partial 16S rRNA gene for the phylogenetic analysis by amplifying the gene directly from cecal samples followed by 454 pyrosequencing for comprehensive analysis of the gut microbiota in broiler chickens. We show that there is a significant difference in the microbial communities between the 2 groups, suggesting the composition of microbial community plays an important role in increased BW and energy harvest in the host.

MATERIALS AND METHODS

Experimental Design for Chicken Study

Two groups with 6 replicate pens of 5 male 1-d-old broiler chicks (Cobb-Vantress Inc., Siloam Springs, AR-72761) per group were fed with the feed supplemented with antibiotics (55 mg/kg; penicillin 100, Alpharma Inc., Bridgewater, NJ) to the test group (ANT) and without antibiotics to the control group (CON). A corn-soybean meal basal diet with essential nutrients supplemented with complete vitamin and trace mineral premixes was used for feeding broiler chicks throughout the study. The birds were weighed at d 1 and at d 18.

Sample Collection and Preparation of DNA Samples

Ceca were dissected from 5 randomly selected birds from each CON and ANT groups. Cecal contents were homogenously suspended in sterile PBS (pH 7.0) for 10-fold dilution (g/mL), finally later pooled together for both the individual groups. Community DNA was isolated from the 10 individual cecal samples as well as from both pooled cecal samples using the Stool DNA Extraction Kit (Qiagen, Valencia, CA), following the manufacturer’s protocol and quantified with NanoDrop 1000 (Thermo Fisher Scientific Inc., Wilmington, DE).

PCR Amplification and Sequencing

Amplification reaction was set up to amplify 16S rRNA gene using universal primers 8F and 515R. Universal primers for 454 pyrosequencing, 454A and 454B, were linked to primers 8F (GCCTTGGCACGCGTCGTA) and 515R (GCCTCCCTCGGCACTCAGCA-GTAT-TACCGGGCTGGCCAC), respectively, at the 5’ end. The PCR mix was made with 1 μL of genomic DNA, 5 μL of 10X enzyme buffer, 4 μL of 2.5 mM dNTP, 3 μL of 25 mM MgCl2, 1 μL each of 350 ng/μL forward and reverse primers, 1 μL of Taq DNA polymerase (New England Biolabs, Ipswich, MA), and double-distilled water to make up a 50-μL reaction mix. Genomic DNA was amplified through a 20 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 60 s. The PCR amplicons were gel-purified and sent for standard GS-FLX sequencing to ICBR (Interdisciplinary Center for Biotechnology Research) Genomics 454 core sequencing laboratories (Genomics Division, University of Florida, Gainesville). Samples were run separately on a 1/16th region plate.

Quantitative PCR

The 16S rRNA gene sequences of Bacteroides thetaiotaomicron ATCC 29741 and Escherichia coli ATCC 33565 were amplified using primers Eub338F (ACTTCCATCCGACGAGGAGG) and Eub1369R (CCGRGAACTTATCCAGC) (Fierer et al., 2005). The amplified products were gel purified using QIAquick Gel Extraction Kit (Qiagen, Valencia, CA) and ethanol precipitated. The purified products were cloned into pGEM-T easy vector (Promega, Madison, WI) followed by transformation into Escherichia coli Top 10. The plasmid with insert from B. thetaiotaomicron 16S rRNA gene amplification was used as the plasmid standard for Bacteroides, and the plasmid with E. coli was used as the standard for Firmicutes. Standard curves were generated with 5 standards in triplicate of 10-fold dilutions. The quantitative PCR (qPCR) reactions were set up in triplicates for standards and 5 samples from each CON and ANT group using primers Buct934F (GGARCATGTG-GTTTAATTGCAGT) and Bact1060R (AGCTGACGACAACCATGC) for Bacteroidetes and Firm934F (GGAGATGTGGTTTATTGCAGA) and Firm1060R (AGCTGACGACAACCATGC) for Firmicutes (Guo et al., 2008) in a final volume of 20 μL per reaction consisting of 10 μL of Power SYBR PCR master mix (Applied Biosystems) with 500 nM of each primer, and 2 ng/μL DNA sample. The reactions were setup for amplifications in StepOne realtime PCR system (Applied Biosystems) as described by Guo et al., 2008.

Data Analysis

Sequence reads obtained were processed using Mothur version 1.22.1 (Schloss et al., 2009) where homopolymers longer than 8 nucleotides, reads shorter than 50 bp, and ambiguous sequences were removed. These sequences were then aligned to Silva reference files 16S rRNA database (Pruesse et al., 2007). Sequences that did not align to 85% of the same region were removed and preclustered followed by removal of chimeric se-
quences followed by analysis with Ribosomal Database Project classifier against its 16S rRNA database (Wang et al., 2007; http://rdp.cme.msu.edu/classifier/classifier.jsp). Ribosomal Database Project results were analyzed using MEGAN version 4.6 (Huson et al., 2007), a metagenomics analysis software.

RESULTS

Microbial Community Analysis

Pyrosequencing generated 7,881 reads for the ANT pool and 11,214 reads from the CON pool with an average read length of 250 bp. Firmicutes was the dominant phylum of the gut community with 58.15% in the CON group versus 91.5% in the ANT group (Figure 1). A shift was also seen in the second dominant phylum Bacteroidetes with a dramatic reduction from 31.1% in CON to 2.96% in the ANT pool. The F/B ratio of the CON group with mean BW of 570.0 g at d 18 was 1.86 and that of the ANT group was 30.92, with a significantly ($P \leq 0.05$) higher mean BW of 668.6 g at d 18. Hence, with the increase in BW, there was also a momentous increase in F/B ratio due to administration of penicillin.

A comparative, phylogenetic tree generated by MEGAN at the genus level illustrated in-depth alteration of the bacterial community (Figure 2). Each circle at the node of the MEGAN tree is scaled logarithmically to represent the normalized number of reads assigned with a pie graph differentiating the 2 groups. Several genera including Prevotella, Bacillaceae, Enterococcus, Lactobacillus, Streptococcus, and Acinetobacter were absent in the ANT group, reducing the diversity in the ANT group compared with CON. A remarkable augmentation in the class Clostridia belonging to phylum Firmicutes was observed attributable to genera Acetanaerobacterium, Moryella, Acetitomaculum, Roseburia, and Acetivivibrio. A percentage decrease in Proteobacteria (inclusive of pathogens such as Escherichia, Salmonella, and Campylobacter) from 8.32% in CON to 3.44% in the ANT group was also noted, an obvious effect of penicillin administration.

qPCR Analysis

The qPCR results obtained from each individuals sample corroborated with pyrosequencing results from the pooled samples, indicating a decrease in F/B ratio from the ANT pool with an average F/B ratio of 35 ± 20.84 to an average F/B ratio of 1.677 ± 0.32 in the CON pool (Figure 3).

DISCUSSION

Gut microflora is a complex and diverse mix of various bacterial phyla with Firmicutes and Bacteroidetes being the dominant ones. Previous researches have established that there is a link between change in BW of animals and alteration in gut microflora (Ley et al., 2005, 2006; Turnbaugh et al., 2006, 2009; Guo et al., 2008). In our study, we observed a similar association in chickens. A significant increase in the BW in relation to an increase in F/B ratio in the penicillin-treated ANT group in comparison with the CON group is evident by both pyrosequencing as well as qPCR analysis. The increase in BW may be due to increased nutrient absorption ability of the chicken caused by thinning of the intestinal wall, a mechanism of action of antibiotics. Diversity in cecal microbiota in chickens, analyzed by a 16S rRNA gene study of broilers, indicated that majority of sequence present belonged to Clostridiaceae (Zhu et al., 2002; Lu et al., 2003), which is in agreement with our phylogenetic analysis that also indicates the dominance of Clostridia, which further increased after penicillin treatment. For growth and development, an elevated amount of energy is required by the body, during which time the F/B ratio is elevated (Mariat et al., 2009). The data reported here support the results in this study that F/B ratio may play an important role in energy uptake and ultimately increased BW. From our results, it can also be inferred that manipulation of gut microbiota can lead to an increase in BW of broiler chickens. The F/B ratio can be potentially used as an indicator for monitoring gut microbiota in chickens as well as a factor for controlling growth performance. Our study warrants research that can help bring about a shift in gut microbiota of chickens that may still lead to the growth-promoting effects without involving AGP.
**Figure 2.** In-depth comparative analysis of taxa from antibiotic (ANT) and control (CON) samples is shown in this MEGAN tree. The nomenclature at each node is based on National Center for Biotechnology Information taxonomy, and the data have been normalized to 100,000 reads per data set.

**Figure 3.** The quantitative PCR data from 10 individual broiler samples from the control (CON) and antibiotic (ANT) groups.
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REFERENCES


