# Bacterial census of poultry intestinal microbiome

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**ABSTRACT** The objective of this study was to generate a phylogenetic diversity census of bacteria identified in the intestinal tract of chickens and turkeys using a naïve analysis of all the curated 16S rRNA gene sequences archived in public databases. High-quality sequences of chicken and turkey gastrointestinal origin (3,184 and 1,345, respectively) were collected from the GenBank, Ribosomal Database Project, and Silva comprehensive ribosomal RNA database. Through phylogenetic and statistical analysis, 915 and 464 species-equivalent operational taxonomic units (defined at 0.03 phylogenetic distance) were found in the chicken and the turkey sequence collections, respectively. Of the 13 bacterial phyla identified in both bird species, *Firmicutes*, *Bacteroidetes*, and *Proteobacteria* were the largest phyla, accounting for >90% of all the sequences. The chicken sequences represent 117 established bacterial genera, and the turkey sequences represent 69 genera. The most predominant genera found in both the chicken and the turkey sequence data sets were *Clostridium*, *Ruminococcus*, *Lactobacillus*, and *Bacteroides*, but with different distribution between the 2 bird species. The estimated coverage of bacterial diversity of chicken and turkey reached 89 and 68% at species-equivalent and 93 and 73% at genus-equivalent levels, respectively. Less than 7,000 bacterial sequences from each bird species from various locations would be needed to reach 99% coverage for either bird species. Based on annotation of the sequence records, cecum was the most sampled gut segment. Chickens and turkeys were shown to have distinct intestinal microbiomes, sharing only 16% similarity at the species-equivalent level. Besides identifying gaps in knowledge on bacterial diversity in poultry gastrointestinal tract, the bacterial census generated in this study may serve as a framework for future studies and development of analytic tools.

**Key words:** 16S rRNA, chicken, naïve analysis, intestinal microbiome, turkey

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## INTRODUCTION

The intestinal track of poultry harbors a complex and dynamic microbial community (or microbiome) consisting primarily of bacteria (Zhu et al., 2002). This microbiome has been recognized to have an important role in host growth performance and health (Brisbin et al., 2008; Yegani and Korver, 2008; Jankowski et al., 2009). The bacteria present in this microbiome can be categorized as commensal or pathogenic bacteria, both of which can be affected by a range of factors, such as host, litter management, diet, and feed additives. Numerous efforts, especially dietary intervention and litter management, have been attempted to modulate this intestinal microbiome to enhance feed conversion and gut health (Owens et al., 2008; Ruiz et al., 2008; Yegani and Korver, 2008). Although limited success has been achieved, few of these interventions have achieved consistent or sustainable improvement. It is now recognized that a better understanding of the interactions of intestinal microbiome with the host and with ingested feed is required to further enhance poultry nutrition and gut health. However, the lack of sufficient knowledge on the bacterial diversity (both phylogenetic and functional) in poultry intestines is considered one of the major knowledge gaps that hinder understanding of such interactions.

The composition and diversity of poultry intestinal microbiome, like other microbiomes, were primarily investigated using cultivation-based methodologies (Barnes et al., 1972; Salanitro et al., 1974) until 16S rRNA gene-targeted analyses were applied in early 2000s (Gong et al., 2002; Zhu et al., 2002). In addition to pathogenic bacteria, these cultivation-based studies helped identify some culturable commensal bacteria, especially facultative anaerobic and aerotolerant anaerobic bacteria. However, it soon became evident that only some of the intestinal bacteria can be cultured in laboratory media (Barnes et al., 1972; Salanitro et al., 1974). The use of DNA-based molecular biology techniques, primarily cloning and sequencing of the 16S


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rRNA gene, have provided opportunities to characterize the unculturable members of intestinal microbiomes of poultry, primarily chickens and turkeys because of practical considerations (Gong et al., 2002; Zhu et al., 2002; Bjerrum et al., 2006; Scupham, 2007a,b; Lu and Domingo, 2008; Scupham et al., 2008). These studies revealed a more complex and diverse intestinal microbiome than previously thought and greatly expanded the perspective on the poultry intestinal microbiome in terms of species composition, diversity, and community structure.

Until recently, all the 16S rRNA gene sequence data sets reported were generated using the Sanger DNA sequencing technology. Due to cost restraints, most studies each produced relatively small numbers of sequences (a few hundred or fewer per sample), thus revealing only a small portion of the full diversity present in the intestinal microbiome. Besides the limited depth of coverage of diversity, the scope of these studies was also narrow with respect to numbers of birds sampled, types of diets and dietary additives fed, housing system and litter management used, and geographic regions surveyed. Additionally, some of the recovered 16S rRNA gene sequences have been deposited in public databases but have not been reported in the literature, contributing little to characterizing and understanding the intestinal bacterial diversity of poultry. Furthermore, as shown for the ruminal microbiome (Edwards et al., 2004; Kim et al., 2011), individual studies can bias toward or against certain bacterial phyla due to the methodology used. As such, the knowledge on the intestinal microbiome of chickens and turkeys remains to be fragmented and biased. We hypothesize that the general bacterial diversity of the intestinal microbiome of poultry can be better defined by analyzing all the 16S rRNA gene sequences (both published and unpublished) collected from all the intestinal microbiomes ever analyzed worldwide. In this study, we performed a naïve analysis of all the publically available 16S rRNA gene sequences that were generated with the Sanger DNA sequencing technology from intestinal samples of both chicken and turkey. We also estimated the current coverage of the bacterial diversity already identified in these 2 domesticated bird species and identified particular gaps in knowledge and understanding of the bacterial populations in these birds. Finally, the bacterial composition was compared between chickens and turkeys.

## MATERIALS AND METHODS

### Sequence Data Collection

The 16S rRNA gene sequences of chicken and turkey origin were retrieved from the 3 public databases of nucleic acids including GenBank (http://www.ncbi.nlm.nih.gov/), Silva comprehensive ribosomal RNA database (Silva, http://www.arb-silva.de/), and Ribosomal Database Project (**RDP**, http://rdp.cme.msu.edu/) in January and February, 2012, using the following search terms: chicken, chickens, chick, chicks, poultry, broiler, hen, hens, turkey, and turkeys. The sequences for chickens and turkeys were downloaded separately. Sequences shorter than 250 bp were removed from the data set to avoid uncertainties in comparing and classifying short sequences that have little or no sequence overlap. Possible chimeric sequences were identified using Chimera Slayer and UCHIME in the Mothur package (Schloss et al., 2009; Edgar et al., 2011; Haas et al., 2011) and removed. The database record information associated with each of the sequences was examined, and the sequences not of poultry gut origin were removed manually. The final sequence data sets were deposited at the MG-RAST server (http://metagenomics.anl.gov/) and accessible through the project Poultry_Gut_DB (4507779.3 to 4507782.3).

### Phylogenetic Diversity Analysis

All the sequences that satisfied the above criteria were aligned using the sequence aligner in Mothur (V1.22) with the Silva SSU_Ref_NR_108 data set (Ludwig et al., 2004) as reference sequences (Schloss et al., 2009). Sequences that could not be aligned due to short overlap with the Silva reference sequences were removed. To generate a detailed phylogenetic tree using the neighbor-joining method (Saitou and Nei, 1987), the resultant aligned sequences were inserted into the Silva ARB tree constructed from the Silva reference data set, with each sequence being inserted into a branch with which the sequence had the greatest sequence similarity. The sequences used in this study is maintained in an in-house ARB database dedicated to the intestinal microbiome of chickens and turkeys and is available from the corresponding author. Krona charts (Ondov et al., 2011) were generated from the sequences for chicken and turkey (and their cecum) using the MG-RAST server (Meyer et al., 2008) to illustrate the composition of intestinal microbiomes of chickens and turkeys. A genus-level taxonomy tree each was also constructed for the sequences from the ceca of chicken and turkey using the MG-RAST server (Meyer et al., 2008) to compare the 2 cecal microbiomes.

### Diversity Estimates

To minimize fragment effect of sequences corresponding to different regions of 16S rRNA gene, the aligned sequences were first clustered based on the Silva bacterial sequence templates using the Cluster.fragment function of Mothur (Schloss et al., 2009). Based on the classifications determined by the Classifier program in Mothur (Wang et al., 2007; Schloss et al., 2009), distance matrices were computed within ARB software (Ludwig et al., 2004) with the Jukes-Cantor correction applied for the following bacterial groups: total bacteria, the phylum *Bacteroidetes*, the phylum *Firmicutes*, the phylum *Proteobacteria*, and sequences of cecum origin.

Separate distance matrices were computed for chickens and turkeys. One distance matrix each was constructed and analyzed at 0.03 (equivalent to species, operational taxonomic unit, **OTU**$_{0.03}$), 0.05 (genus, OTU$_{0.05}$), 0.10 (family), and 0.20 (phylum) phylogenetic distances (Schloss and Handelsman, 2004). The Mothur program (Schloss et al., 2009) was used to cluster sequences into OTU, generate rarefaction curves, and determine the nonparametric ACE and Chao1 estimates of maximum richness from each of the distance matrices. The distance matrices were computed 3 times, and the median was chosen in calculating these indices to avoid under- or overestimation.

The maximum number of OTU present in the intestinal and cecal microbiomes of each bird species was estimated using the nonlinear procedure (PROC NLIN) of SAS (V9.2, SAS Inst. Inc., Cary, NC). This method fits the monomolecular function to the rarefaction output to determine the asymptote that serves as the upper bound of the curves as previously described (Larue et al., 2005). The value defined by the asymptote is an estimate of the expected maximum species richness complementary to the ACE and Chao1 richness estimates and has been used previously to estimate maximum species richness in different types of microbiomes (Larue et al., 2005; Youssef and Elshahed, 2008; Nelson et al., 2010; Kim et al., 2011). The percent coverage was calculated by dividing the observed number of OTU by the maximum number of OTU (Kim et al., 2011). The number of sequences that would be required to provide 99% coverage at 0.03 and 0.05 phylogenetic distances was estimated using the same nonlinear model (Larue et al., 2005; Kim et al., 2011).

### Comparison of Intestinal Microbiomes Between Chickens and Turkeys

The intestinal microbiomes of chickens and turkeys were compared using 3 methods: weighted UniFrac distance, which measures the phylogenetic distance between sets of taxa as phylogenetic trees (Lozupone and Knight, 2005; Lemos et al., 2012); the SONS function in the Mothur package, which compares 2 microbiomes by taking into consideration of OTU richness, membership, and structure (Schloss and Handelsman, 2006; Schloss et al., 2009); and Krona charts that allows comparison between micobiomes based on detailed phylogenetic composition. The cecal microbiomes between chicken and turkey were compared on a RDP annotated taxonomy tree at genus level using the MG-RAST server.

## RESULTS AND DISCUSSION

In total, 33,598 16S rRNA gene sequences of chicken and turkey gut origin were retrieved from GenBank, RDP, and Silva databases using the search terms. Of these sequences, 3,184 from chickens and 1,345 from turkeys passed the selection criteria and were analyzed in this study (Table 1), reflecting a fact that more than 85% of the 16S rRNA gene sequences archived in public databases are of short length or poor quality, or without a clear record of poultry gut as the sampling location. These sequences represent 13 existing bacterial phyla, besides 5.3 and 6.8% of the chicken and the turkey sequences, respectively, that could not be classified to any of the phyla within the Bergey's taxonomy implemented in the RDP database (Figures 1 and 2). The sequences of chicken origin were assigned to 915 species-equivalent OTU$_{0.03}$ within 655 genus-equivalent OTU$_{0.05}$, whereas the sequences recovered from turkeys were grouped into 464 OTU$_{0.03}$ within 364 OTU$_{0.05}$. The sequences from chicken gut represented 12 existing phyla of bacteria (Figure 1), while the sequences from turkey gut represented 8 recognized bacterial phyla (Figure 2). Compared with the gut microbiome of other animals, the numbers of sequences recovered from both chickens and turkeys, and the diversity represented by these sequences, are relatively small. The fast transit and thus short retention time in the poultry gut (approximately 4 h for chickens) might be a major reason for such relatively low diversity.

### The Global Diversity of Intestinal Microbiome Sampled from Chickens

Of the 12 phyla of bacteria represented by the 3,184 high-quality 16S rRNA gene sequences of chicken origin, *Firmicutes* was the most predominant phylum and accounted for almost 70% of all the bacterial sequences of chicken origin (Figure 1). The *Firmicutes* sequences were grouped into 713 OTU$_{0.03}$ within 495 OTU$_{0.05}$ (Table 1). *Bacteroidetes* (12.3% of the bacterial sequences) and *Proteobacteria* (9.3% of the bacterial sequences) were the second and third most predominant phyla, represented by 172 and 157 OTU$_{0.03}$ within 139 and 124 OTU$_{0.05}$, respectively. Other minor phyla were only represented by a small number of OTU, each of which was represented by small numbers of sequences. The predominance of *Firmicutes* documented in the chicken gut was much greater, whereas that of *Bacteroidetes* was smaller, than in the gut of other domesticated food animals sampled.

In total, 117 established genera of bacteria were represented by the sequence collection, with most genera belonging to the phyla *Firmicutes*, *Proteobacteria*, and *Bacteroidetes* (Figure 1). However, most of these genera were represented by a small number of sequences. Within phylum *Firmicutes*, genera *Clostridium*, *Ruminococcus*, *Lactobacillus*, *Eubacterium*, *Fecalibacterium*, *Butyrivibrio*, *Ethanoligenens*, *Alkaliphillus*, *Butyricicoccus*, *Blautia*, *Hespellia*, *Roseburia*, and *Megamonas* were represented by more than 1% of the total bacterial sequences (in descending order). Most of these predominant genera are common intestinal residents, but the relatively high prevalence of *Ethanoligenens*, a genus of

**Table 1.** The number of operational taxonomic units (OTU) for predominant bacterial phyla and groups, their percentage coverage, diversity index, and number of sequences needed to reach 99% coverage

| Bacterial phyla or groups | No. of sequences | Observed no. of OTU (% coverage) | | Maximum no. of OTU: Rarefaction asymptote | | Maximum no. of OTU: Chao1 | | Maximum no. of OTU: ACE | | No. of sequences needed to reach 99% asymptote | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 0.03 | 0.05 | 0.03 | 0.05 | 0.03 | 0.05 | 0.03 | 0.05 | 0.03 | 0.05 |
| Chicken | | | | | | | | | | | |
| Total bacteria | 3,184 | 915 (89) | 655 (93) | 1,028 | 703 | 904 | 791 | 968 | 834 | 6,843 | 5,982 |
| Bacteroidetes | 391 | 172 (58) | 139 (66) | 296 | 212 | 497 | 289 | 686 | 466 | 2,142 | 1,753 |
| Firmicutes | 2,192 | 713 (84) | 495 (90) | 856 | 551 | 947 | 662 | 1,025 | 700 | 5,821 | 4,771 |
| Proteobacteria | 295 | 157 (38) | 124 (54) | 415 | 230 | 628 | 316 | 660 | 560 | 2,928 | 1,799 |
| Cecal bacteria | 972 | 532 (63) | 400 (76) | 846 | 530 | 785 | 638 | 903 | 901 | 4,597 | 3,297 |
| Turkey | | | | | | | | | | | |
| Total bacteria | 1,345 | 464 (68) | 364 (73) | 681 | 497 | 984 | 917 | 1,136 | 1,168 | 5,652 | 4,978 |
| Bacteroidetes | 387 | 99 (60) | 90 (65) | 167 | 138 | 401 | 319 | 578 | 617 | 2,071 | 1,771 |
| Firmicutes | 812 | 294 (70) | 213 (76) | 423 | 280 | 705 | 507 | 961 | 617 | 3,292 | 2,796 |
| Proteobacteria | 80 | 29 (77) | 24 (79) | 38 | 31 | 47 | 41 | 67 | 54 | 262 | 250 |
| Cecal bacteria | 958 | 350 (59) | 275 (68) | 596 | 405 | 765 | 734 | 1,151 | 964 | 5,137 | 4,049 |

ethanol-producing bacteria, is intriguing. Within phylum *Proteobacteria*, genus *Desulfohalobium* was represented by the most sequences (0.7% of the bacterial sequences), and within phylum *Bacteroidetes* most of the sequences were classified into order *Bacteroidales*, and only genera *Bacteroides*, *Prevotella*, *Parabacteroides*, and *Alistipes* were each represented by >1% of the bacterial sequences. Of the minor phyla, *Actinobacteria* was the most predominant, but only the genus *Bifidobacterium* was represented by >1% of sequences within this phylum (Figure 1). Other minor phyla, including *Cyanobacteria*, *Spirochaetes*, *Synergistetes Fusobacteria*, *Tenericutes*, and *Verrucomicrobia*, were only represented by no more than several sequences, suggesting their low abundance or prevalence in the gut of chickens. As for archaea, only phylum *Euryarchaeota* was represented by a very small number of sequences, corroborating low abundance or prevalence of methanogens in the gut of chickens (Saengkerdsub et al., 2007).

The numbers of OTU clustered at different phylogenetic distances were examined using rarefaction analysis (Supplemental Figure 1A, available online at http://ps.fass.org/). At 0.03 phylogenetic distance or more, the rarefaction curves approached plateau, but continued to project upward at <0.03 distance. These results suggest that the diversity at the subspecies level has not been completely sampled. The parametric (rarefaction) and nonparametric (Chao1 and ACE) estimates of richness were similar (Table 1). Based on these estimates, 904 to 1,028 bacterial $OTU_{0.03}$ and 703 to 834 $OTU_{0.05}$ likely exist in the gut of chickens collectively. As in the case of the observed OTU, most of the predicted OTU were within phylum *Firmicutes*. Based on the parametric estimate, the data set of this study has documented at least 89% of the $OTU_{0.03}$ and 93% of the $OTU_{0.05}$, and approximately 6,800 additional sequences from multiple chicken flocks in different geographic regions would probably allow for identification of 99% of the $OTU_{0.03}$ (Table 1), with most of the sequences to be recovered from members of *Firmicutes*. The acquisition of these new sequences will probably reveal all the $OTU_{0.05}$.

## The Global Diversity of Intestinal Microbiome Sampled from Turkeys

The 1,345 bacterial 16S rRNA gene sequences of turkey gut origin represented 8 phyla of bacteria, and 93.2% of these sequences were classified to existing phyla (Figure 2). The most predominant phyla included *Firmicutes* and *Bacteroidetes*, accounting for approximately 60.4 and 28.8% of the total sequences from turkeys, respectively. Except *Proteobacteria* and *Actinobacteria*, each of the other minor phyla was represented by only a small number of bacterial sequences. The turkey sequences were grouped into 464 $OTU_{0.03}$ within 364 $OTU_{0.05}$ (Table 1), as in the case of chicken sequences, most of which were found within *Firmicutes* and *Bacteroidetes*. However, phylum *Bacteroidetes* was represented by a higher proportion of total bacterial sequences in turkeys than in chickens. The increased proportion of *Bacteroidetes* was at the expense of that of *Firmicutes*. Because the diets between domesticated chickens and turkeys are quite similar, the above differences in gut bacterial diversity might be mainly attributed to host differences.

The taxonomic composition of the turkey bacteria was detailed at the genus level in the Krona chart (Figure 2). The turkey sequence data set identified 69 genera of bacteria; however, 20 of them were singletons (Figure 2). *Firmicutes* alone was represented by 37 genera, but only *Ruminococcus*, *Clostridium*, and *Lactobacillus* each represented more than 5% of all the sequences of this phylum. Other genera that was represented by more than 1% of the total bacterial sequences included (in descending order): *Megamonas*, *Bacillus*, *Fecalibacterium*, *Virgibacillus*, *Blautia*, *Eubacterium*, *Butyrivibrio*, *Ethanoligenes*, *Butyricoccus*, and *Clostridiales* family XI Incertae Sedis. *Bacteroides* was the most predominant genus, accounting for 79% of the sequences, in the

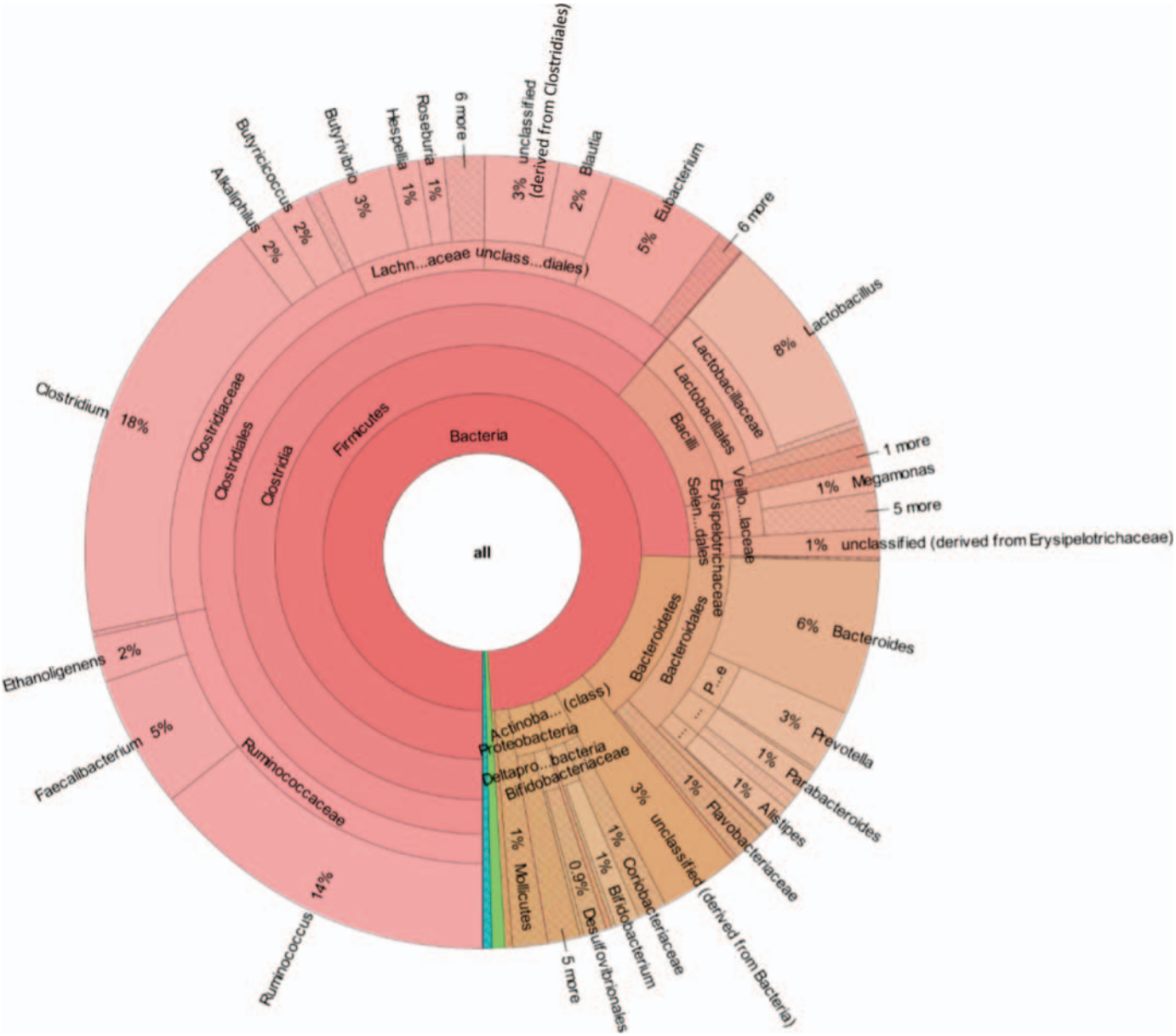


**Figure 1.** Krona chart of the bacteria represented by 16S rRNA sequences recovered from chicken gut (from 3,030 of the 3,184 total chicken sequences). Color version available in the online PDF.

phylum *Bacteroidetes*. Other relatively predominant genera in this phylum included *Prevotella* and *Paraprevotella*. Within phylum *Proteobacteria*, *Desulfohalobium* and *Aeromonas* were the most predominant genera.

The numbers of OTU observed at phylogenetic distances $\geq$0.03 tended to approach plateau, but not at <0.03 distance (Supplemental Figure 1B, available online at http://ps.fass.org/). Unlike in the case of the chicken sequences, the Chao1 and ACE estimates of richness were greater than the parametric rarefaction estimate for most of the bacterial groups (Table 1). Based on the rarefaction estimate, at least 681 $OTU_{0.03}$ within 497 $OTU_{0.05}$ might be found in the gut of turkeys collectively, with most of them being within phyla *Firmicutes* and *Bacteroidetes*. The sequence data set of turkey provided lower coverage than that of chicken because of the smaller number of sequences that have been recovered from turkeys. To achieve 99% coverage of diversity at phylogenetic distance 0.03, at least 5,652 sequences might need to be collected from multiple turkey flocks.

## The Global Diversity of Intestinal Microbiome Sampled from Chicken Cecum

The sampling locations of chicken gastrointestinal tract were not all clearly documented in the databases. Among the 16S rRNA gene sequences annotated with sampling locations, 972 were sampled from chicken cecum. These sequences represented 10 known bacterial phyla and accounted for 92.8% of the chicken cecal sequences (Figure 3). The most predominant phyla

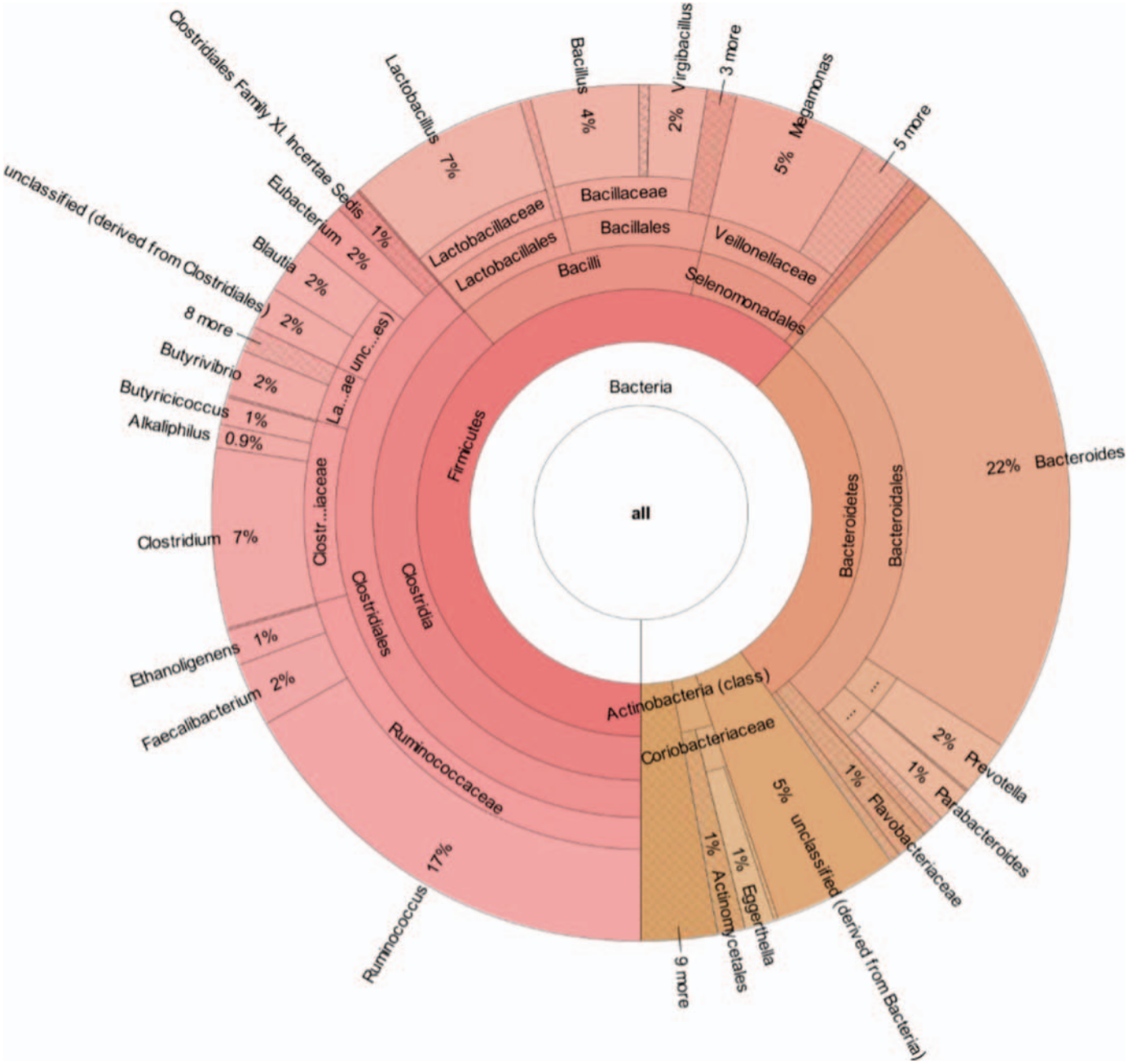


**Figure 2.** Krona chart of the bacteria represented by 16S rRNA sequences recovered from turkey gut (from 1,254 of the 1,345 total turkey sequences). Color version available in the online PDF.

included *Firmicutes* and *Bacteroidetes*, accounting for approximately 78 and 11% of the total cecal sequences, respectively. Except for *Proteobacteria* and *Actinobacteria*, the other minor phyla each were represented by only a small number of bacterial sequences. The sequences from chicken cecum were grouped into 532 $OTU_{0.03}$ within 400 $OTU_{0.05}$ (Table 1).

The cecal sequences from chicken identified 59 bacterial genera; however, 26 of them were represented by only a single sequence (Figure 3). *Firmicutes* alone contained 31 genera, but only *Ruminococcus*, *Clostridium*, and *Eubacterium* each represented ≥5%, of the sequences classified to this phylum. Other genera that contained more than 1% of the total cecal bacterial sequences included (in descending order): *Fecalibacterium*, *Blautia*, *Butyrivibrio*, *Lactobacillus*, *Megamonas Roseburia*, *Ethanoligenes*, *Hespellia*, *Veillonella*, and *Anaerostipes*. *Bacteroides* was the most predominant genus in the phylum *Bacteroidetes*, accounting for 40% of the cecal sequences in this phylum. Other relatively predominant genera in this phylum included *Prevotella* and *Paraprevotella*, *Tanneralla*, and *Riemeralla*. Within phylum *Proteobacteria*, *Desulfohalobium*, *Escherichia/Shigella*, and *Neissenia* were the most predominant genera.

The numbers of $OTU_{0.03}$ tended to approach plateau, but not the number of unique sequences (Supplemental Figure 1C, available online at http://ps.fass.org/).

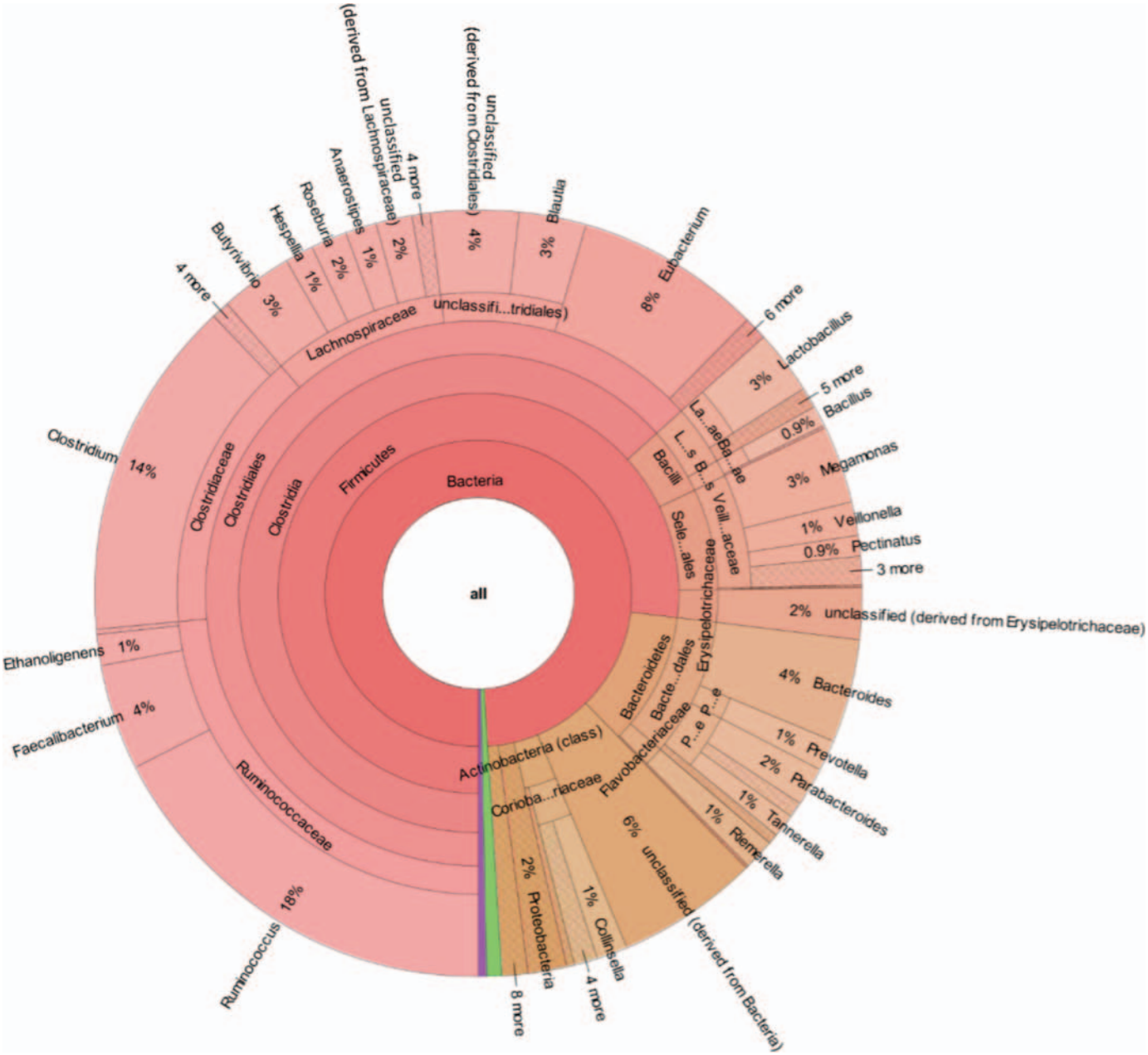


**Figure 3.** Krona chart of bacteria from chicken cecum (from 919 of the 972 chicken cecal sequences). Color version available in the online PDF.

Based on the rarefaction, Chao1 and ACE estimates, 785 to 903 $OTU_{0.03}$ within 530 to 901 $OTU_{0.05}$ might be found in the cecal microbiome of chicken collectively, with most of them being within phyla *Firmicutes* and *Bacteroidetes*. The diversity coverage for chicken cecum was lower than that for the entire chicken gut because of the smaller number of sequences that have been recovered from the cecum. To achieve 99% coverage of the diversity at the species-equivalent level, at least 4,597 sequences might need to be collected from multiple flocks.

The bacterial diversity present in the chicken cecum has been investigated recently using 454 pyrosequencing (Qu et al., 2008; Callaway et al., 2009; Lee et al., 2011; Stanley et al., 2012a,b). The massive parallel sequencing capacity of this technology allows for deeper coverage of diversity than the Sanger sequencing technology. The bacterial profiles revealed in chicken cecum varied considerably among these studies with respect to number of OTU and genera detected and their relative proportion. Even so, all the genera that have been identified from the 454 pyrosequencing of 16S rRNA gene amplicons (Callaway et al., 2009; Lee et al., 2011; Stanley et al., 2012a,b) were represented in the global sequence data set. Therefore, even though the coverage of the individual studies was low, the global sequence data set represents much of the diversity present in chicken cecum and can serve as a phylogenetic framework of the bacterial diversity of chicken cecum. The global sequence data set of the chicken cecum was also compared with the 16S sequences recovered from chicken cecum by shotgun pyrosequencing (Qu et al.,

2008) on the MG-RAST server. When the global sequence database of chicken cecal bacteria identified 59 bacterial genera, the shotgun pyrosequencing data set only detected 21 bacterial genera, and 7 of them (*Corynebacterium*, *Paracoccus*, *Helicobacter*, *Trabulsiella*, *Candidatus phytoplasma*, and *Akkermansia*) were not represented in the global sequence data set. This might reflect the bias of individual studies that hindered a comprehensive knowledge of composition of the intestinal microbiome.

The predominant genera represented in the global sequence data set (of chicken cecum origin) also differed from those identified by 454 pyrosequencing studies. *Ruminococcus*, *Lactobacillus*, and *Bacteroides* were the most predominant genera in the global sequence data set and in two 454 pyrosequencing studies (Qu et al., 2008; Stanley et al., 2012a). However, *Bacteroides* and *Prevotella* were found to be the most predominant genera in the chicken cecum by Callaway et al. (2009), whereas *Butyricimonas* and *Fecalibacterium* were more predominant than other genera in the study by Nordentoft et al. (2011). The relative abundance of *Lactobacillus*, *Clostridium*, and *Ruminococcus* in the global sequence data set of chicken cecum was 3, 14, and 18%, respectively, whereas their relative abundance ranged from <2 to >20% among the 454 pyrosequencing studies (Qu et al., 2008; Callaway et al., 2009; Nordentoft et al., 2011; Stanley et al., 2012a,b). Differences in host, feed, and biases associated with the analysis techniques used might all contribute to the discrepancy. Thus, comparison of the relative abundance of individual genera or OTU among different studies should be interpreted with caution. All the major enteric pathogenic bacteria were represented in the global sequence data set, but *Campylobacter* and *Shigella* were not detected in any of the 454 pyrosequencing data sets.

## The Global Diversity of Intestinal Microbiome Sampled from Turkey Cecum

Among the sequences annotated with sampling locations, 958 bacterial 16S rRNA gene sequences were sampled from turkey cecum. Most of these sequences (99.8%) were assigned to 7 bacterial phyla (Figure 4). The most predominant phyla included *Firmicutes* and *Bacteroidetes*, accounting for approximately 55 and 37% of the total turkey cecal sequences, respectively. As in the case of chicken cecal microbiome, except for *Proteobacteria* and *Actinobacteria*, the other minor phyla were each represented by a small number of bacterial sequences. The sequences from turkey cecum were grouped into 350 $OTU_{0.03}$ within 275 $OTU_{0.05}$ (Table 1).

The sequences from turkey cecum identified 50 bacterial genera, 15 of which were represented by only a single sequence (Figure 4). In the phylum *Firmicutes*, genera *Ruminococcus*, *Clostridium*, *Fecalibacterium*, and *Megamonas* each represented ≥5% of the turkey cecal sequences, whereas genera *Blautia*, *Butyrivibrio*, *Butyricoccus*, *Alkaliphillus*, *Eubacterium*, and *Pectinatus* were each represented by ≥1% of the *Firmicutes* sequences (in descending order). *Bacteroides* was the most predominant genus, accounting for 80% of the *Bacteroidetes* sequences of turkey cecum. Other relatively predominant genera in this phylum included *Prevotella* and *Paraprevotella*. The remaining phyla were each represented by only several sequences.

The numbers of $OTU_{0.03}$ identified in turkey cecum tended to approach plateau, but not the number of unique sequences (Supplemental Figure 1D, available online at http://ps.fass.org/). Based on the rarefaction estimation, at least 596 $OTU_{0.03}$ within 400 $OTU_{0.05}$ might be found in the cecum of turkeys collectively. As in the case of chicken cecum, the bacterial diversity coverage for turkey cecum was lower than that for the entire turkey gut because of the small number of sequences that have been recovered. To achieve 99% coverage of bacterial diversity at species-equivalent level, at least 5,137 sequences need to be collected from multiple flocks.

## Comparisons of Global Diversity of Intestinal Microbiome Between Chickens and Turkeys

The intestinal microbiomes of chickens and turkeys represented by the composite sequence data sets analyzed in this study appeared to be significantly different based on UniFrac significance analysis and $P$ test ($P < 0.01$). When compared with respect to OTU richness, membership, and structure using the SONS function within the Mothur program, the Yue-Clayton similarity index ($\theta_{yc}$, ranging from 0 for 2 completely different communities to 1 for 2 identical communities; Yue and Clayton, 2005) was only 0.16 at species-equivalent level and 0.59 at phylum level, indicating 2 distinct intestinal microbiomes (Table 2). The 2 intestinal microbiomes were more similar with respect to phylum *Proteobacteria*, followed by *Firmicutes*. On the other hand, the 2 microbiomes shared little similarity with respect to phylum *Bacteroidetes*. As expected, greater community similarities were shared at higher phylogenetic distances. Noticeably, the cecal microbiomes of the 2 bird species shared a lower $\theta_{yc}$ similarity index at each difference level compared with the total gut intestinal microbiomes, suggesting that the cecal microbiomes of the 2 bird species were also distinct, and the microbiomes in other intestinal segments might share a relatively higher similarity between the 2 birds (Table 2). However, an in-depth analysis of the microbiomes from other gut sections was not feasible because only small numbers of sequences have been recovered from other intestinal segments of these 2 species.

Recently, Lemos et al. (2012) noted that OTU-based approaches, when applied to data sets with low sequence coverage, may lack the resolution to detect

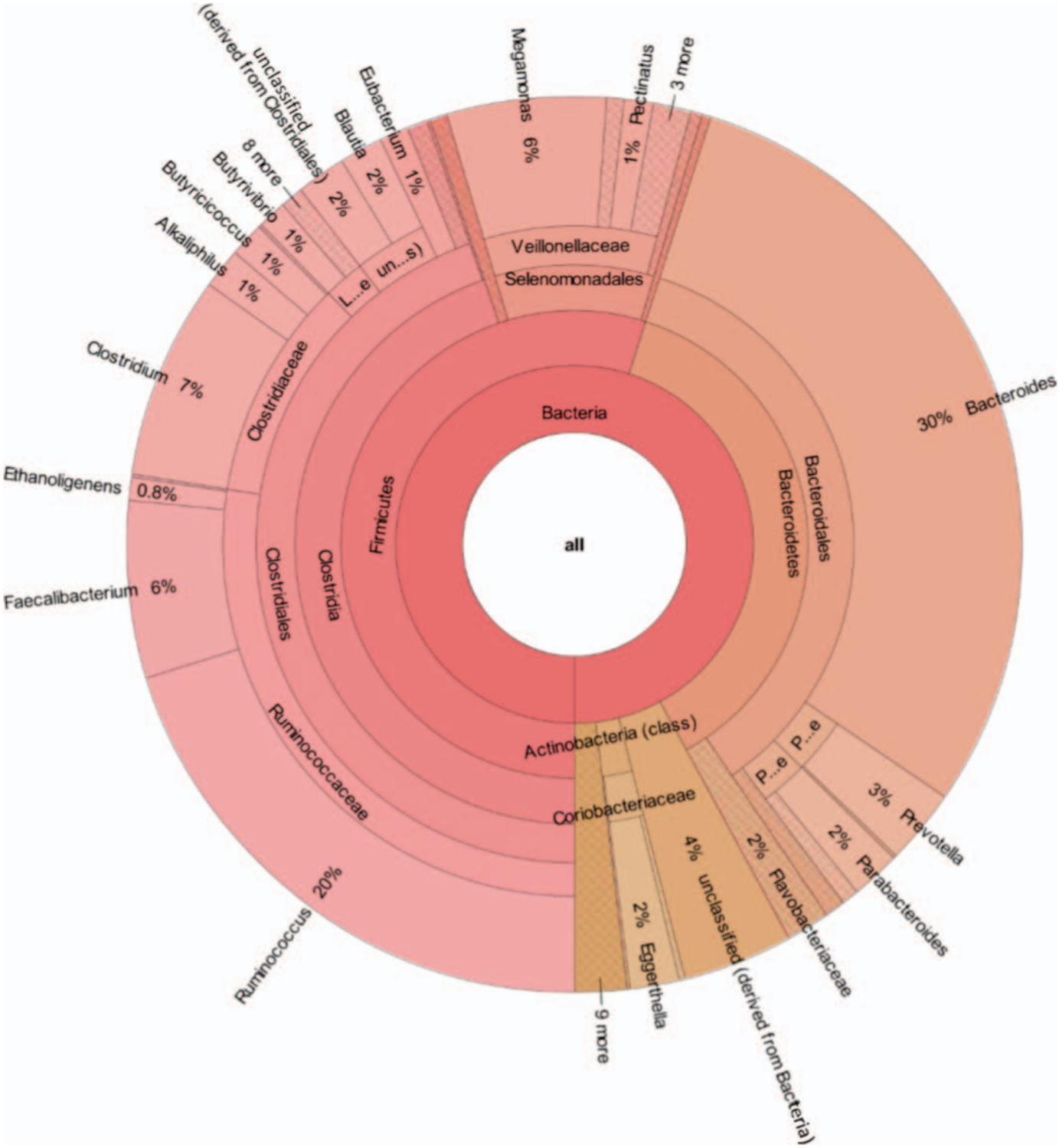


**Figure 4.** Krona chart of bacteria from turkey cecum (from 956 of the 958 turkey cecal sequences). Color version available in the online PDF.

overlapping species between microbiomes. On the other hand, weighted UniFrac distances was suggested to be a reliable index when comparing both the diversity and structure of bacterial communities for all sequencing data sets, even the ones with a relatively small number of sequences. Comparison using weighted UniFrac distances revealed that the intestinal microbiomes of chickens and turkeys shared less than 50% overall similarity in diversity and phylogenetic structure (Table 2), and *Proteobacteria* was the phylum that was shared the most between the 2 bird species, which agreed with the results of the SONS analysis.

Besides differences in sequence predominance between the intestinal microbiomes of the 2 bird species, the distribution and relative abundance of the bacterial genera in the cecum also differed between chicken and

**Table 2.** Comparisons of intestinal bacterial diversity between chickens and turkeys

| Source | Distance level | No. of OTU shared | $\theta_{yc}$[1] (lci, hci) | UniFrac distance |
|---|---|---|---|---|
| Total bacteria | | | | |
| | 0.03 | 191 | 0.161 (0.130, 0.191) | 0.598 |
| | 0.05 | 180 | 0.187 (0.154, 0.220) | 0.596 |
| | 0.20 | 25 | 0.587 (0.544, 0.630) | 0.721 |
| *Bacteroidetes* | | | | |
| | 0.03 | 14 | 0.085 (0.041, 0.129) | 0.736 |
| | 0.05 | 19 | 0.115 (0.064, 0.165) | 0.754 |
| | 0.20 | 4 | 0.631 (0.548, 0.714) | 0.946 |
| *Firmicutes* | | | | |
| | 0.03 | 144 | 0.239 (0.188, 0.291) | 0.567 |
| | 0.05 | 131 | 0.307 (0.251, 0.364) | 0.570 |
| | 0.20 | 14 | 0.756 (0.711, 0.802) | 0.630 |
| *Proteobacteria* | | | | |
| | 0.03 | 13 | 0.416 (0.267, 0.564) | 0.524 |
| | 0.05 | 12 | 0.473 (0.309, 0.637) | 0.611 |
| | 0.20 | 6 | 0.762 (0.642, 0.881) | 0.814 |
| Cecal | | | | |
| | 0.03 | 91 | 0.081 (0.052, 0.111) | 0.656 |
| | 0.05 | 88 | 0.117 (0.082, 0.152) | 0.690 |
| | 0.20 | 20 | 0.374 (0.324, 0.424) | 0.738 |

[1] $\theta_{yc} = \frac{\sum_{i=1}^{S_T} a_i b_i}{\sum_{i=1}^{S_T} (a_i - b_i)^2 + \sum_{i=1}^{S_T} a_i b_i}$ (Yue and Clayton, 2005), where $S_T$ = the total number of operational taxonomic units (OTU) in communities A and B; $a_i$ = the relative abundance of OTU i in community A; $b_i$ = the relative abundance of OTU i in community B. lci, lower CI (95%); hci, higher CI (95%).

turkey (Figure 5). Although the cecal microbiomes of both bird species shared the major phyla, *Actinobacteria*, *Bacteriodetes*, *Firmicutes*, and *Proteobacteria*, they showed distinct distribution and relative abundance at genus level. One extreme example is in *Actinobacteria* in which no genus was shared between the 2 bird species. More genera of *Bacteroidetes* were found in the turkey cecal microbiome, and 6 of them (*Porphyromonas*, *Paraprevotella*, *Capnocytophaga*, *Elizabethkingia*, *Flavobacterium*, and *Ornithobacterium*) were not found in the cecal microbiome of chicken. On the other hand, the chicken cecal microbiome contained more genera of *Proteobacteria*, and 9 of them were not found in the cecum of turkey. Being the most predominant phylum in both bird species, *Firmicutes* consisted of 42 genera, but only 24 genera were shared by both bird species, leaving 13 genera exclusively found in the chicken cecum and 5 genera only identified in the turkey cecum.

Differences in host (genetics, breeds, anatomical features of gut, physiology, and so on) and feeds may be attributable to the observed differences in bacterial diversity between these 2 bird species. For examples, the intestines are larger in diameter in turkeys than in chickens. It is also known that turkeys have a more viscous digesta and a slower digesta passage rate (i.e., longer retention time) than chickens (Palander et al., 2010). These factors may result in lower partial $O_2$ pressure and redox potential in the gut of turkeys than in the gut of chickens. These factors may explain, at least partially, the greater predominance of *Bacteroides* and *Fecalibacterium*, which are 2 strictly anaerobic genera predominant in the gut of mammalian animals, but smaller predominance in facultatively anaerobic genera, such as *Enterococcus*, *Streptococcus*, *Blautia*, *Subdoligranulum*, and several unclassified bacteria, in the gut of turkeys than in the gut of chickens (Figures 3, 4, and 5). Further, domesticated turkeys are grown primarily in the United States and Canada, and most of the turkey sequences in the sequence data set were generated in several comprehensive studies conducted in the United States (Scupham, 2007a,b; Lu and Domingo, 2008; Scupham et al., 2008). The narrower geographic regions of turkeys than chickens that have been sampled might also contribute to the observed differences in the 2 microbiomes. The differences in intestinal microbiome between these 2 bird species have important implications. Approaches to manipulate the intestinal microbiome may not be equally applicable to both species of birds. Indeed, chicken-derived competitive exclusion cultures effectively protected chicks, but not young turkeys, from infection with *Salmonella kedougou* or *Salmonella typhymurium* (Hollister et al., 1994). It should also be noted that almost all the turkey sequences were represented by uncultured bacteria, reflecting the lack of cultivation-based studies on the intestinal bacteria in turkeys.

## Toward a Comprehensive Perspective of Poultry Intestinal Microbiome

Including sequences recovered from different chickens and turkeys fed different diets in different countries using a range of methodologies, the sequence data set established in the present study can serve as a global phylogenetic framework of bacterial diversity identified in chickens and turkeys. According to the estimates from the sequence data sets, less than 7,000 new sequences each from chickens and turkeys will probably allow

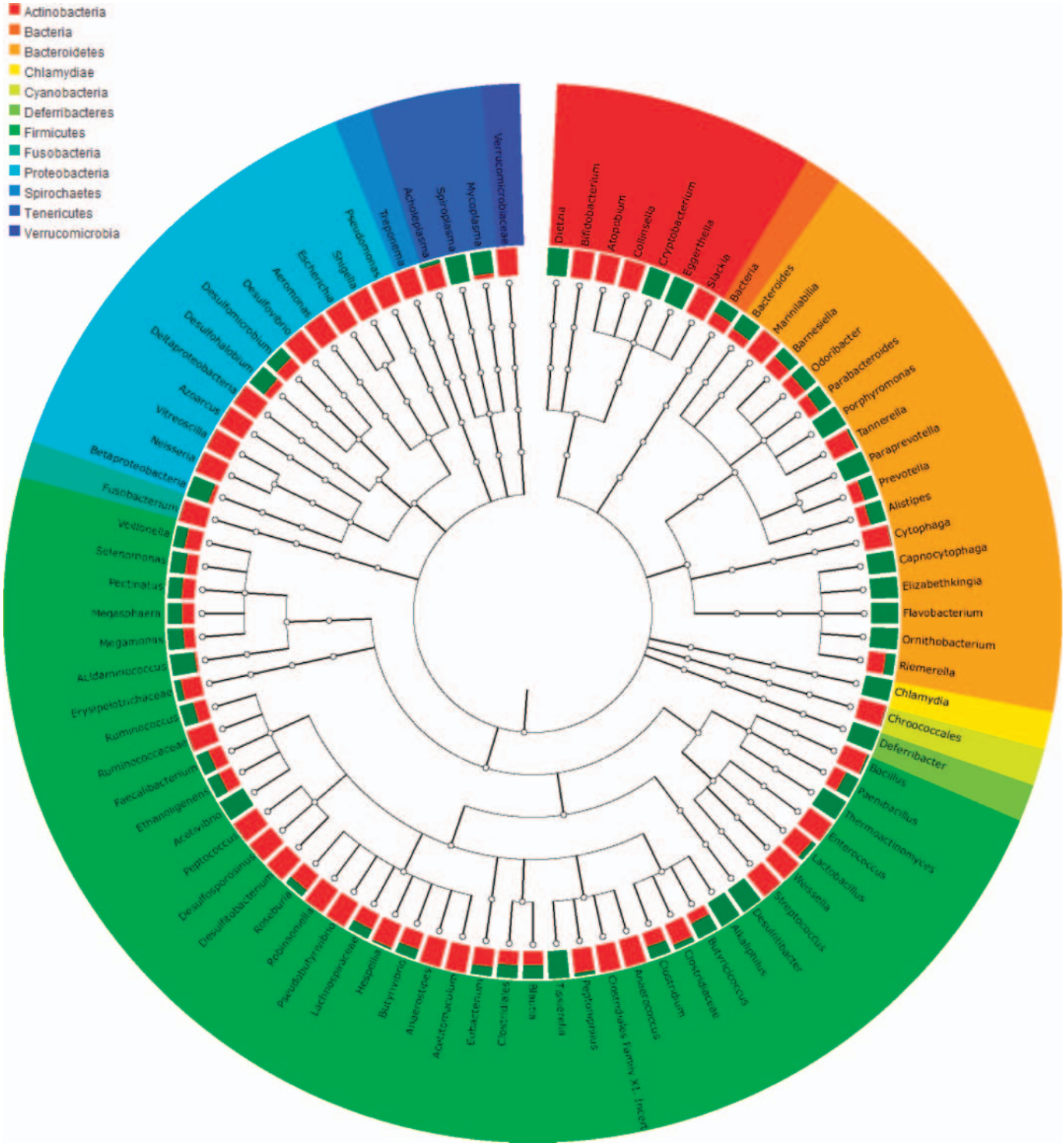


**Figure 5.** Distribution of bacterial genera identified in the cecal microbiomes of chicken and turkey. Red bars: chicken cecum; green bars: turkey cecum.

nearly complete (99%) coverage at both species- and genus-equivalent levels. It should be noted, however, these new sequences need to be recovered from different flocks of chickens and turkeys fed different diets across broad geographic regions. Otherwise, the data will be biased toward one or a few groups of birds fed a few diets in limited regions, and thus the sequence data will not represent true global diversity, even with increased depth of coverage afforded by the next generation DNA sequencing technologies. Furthermore, the current coverage might be an underestimate because with increasing number of sequences the predicted species richness tends to increase (Yu et al., 2006; Roesch et al., 2007). Thus, more than 7,000 new sequences from each bird species might be needed to achieve almost complete coverage of the bacterial diversity. Nevertheless, with

the advancement of next-generation DNA sequencing technologies, it is feasible, both technically and fiscally, to generate sufficient new sequences. Given the bias noted in 454 pyrosequencing profiles, a coordinated effort from researchers is needed to sample chickens and turkeys fed diverse diets from different countries. The knowledge of the full diversity of gut microbiome can provide a diversity framework to assess the significance of individual populations in poultry gut and development of new analytic tools.

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