Original Research Article

Survey of the Intestinal Bacterial Compositions of Three Sympatric Passeriformes Species

ABSTRACT

Aims: The gut microbiota of avian species, especially wild birds, is studied less than that of mammals. Moreover, due to ethical concerns, a vast majority of microbiota studies have been limited to their feces. In this study, we investigated the microbial composition in the upper intestinal tract of sympatric, heterospecific bird species.
Study design: We obtained samples from three wild bird species belonging in the same Order Passeriformes: Cedar Waxwing (*Bombycilla cedrorum*), American Robin (*Turdus migratorius*) and Dark-eyed Junco (*Junco hyemalis*). The samples were obtained from wild birds soon after they died due to physical injury. Microbial diversity was analyzed via high-throughput sequencing of 16S rRNA gene fragments.
Place and Duration of Study: Indiana University Kokomo campus, Indiana. United States, between October 2019 and December 2020.
Methodology: Illumina Miseq sequencing of V3-V4 region of the 16S rRNA gene was employed to determine microbial composition. The α and β diversity were determined by Shannon's diversity index and principal coordinate analysis, respectively. Pair-wise comparison of Bray-Curtis dissimilarity between birds of same species, different species, collection dates and years were analyzed.
Results: The proteobacterial sequences were predominant in all samples. However, the comparison at the level of bacterial class revealed a significant intraspecies variation in both α and β diversity. Additionally, some microbiota exhibited higher interspecies similarity than intraspecies similarity, despite these birds not sharing the same foraging guilds.
Conclusion: Our study revealed high variability in the microbial compositions within the upper part of the digestive tract of closely related Passeriform birds in their natural environment.

Keywords: Gut Microbiota, Cedar Waxwing, American Robin, Dark-eyed Junco, 16S rRNA

1. INTRODUCTION

Gut microbial composition greatly impacts the physiology of the host. The microbiome can play a crucial role in physiological processes, such as digestion, vitamin production, protection against pathogens, as well as maturation of hosts' immune system and brain functions, including those that affect behavior [1,6,10,13,20,25,32].

Diet has been shown to have substantial impact on microbiota of animals, including birds [9,11,17,34]. Some studies have utilized artificial food to study the effects of a single component (e.g., the amount of dietary fiber or fat content) on microbiota [9,35]. Others have examined the impact of specific aspects of diet (e.g., calories) on microbiota [15]. Studying the impact of diet on microbiota of animals in natural environments is challenging, as organisms consume a variety of foods, often with seasonal variations.

Only a few bird species (2%) are strictly herbivorous [7,31], while 32% of all animals are herbivores [31]. Herbivory in birds is most likely rare due to the high metabolic needs of birds and fermentation being a relatively slow process. Many birds rely on insects for at least part of their diets. Many bird species tend to switch their diets after their breeding seasons and/or during the colder months when less arthropod prey is available. During the colder months, their diet often become more frugivorous or granivorous [4,22]. This type of diet switch leads to an increase in gut length and change in microbial composition [5,28,37].

In addition to diet, other environmental and genetic factors can also alter gut microbiota. Hosts' social and physical environments, geographical distance, age, sex, active migration, and foraging strata have all been shown to contribute to the variations of microbiome composition al beit at different degrees [19,30,43]. In particular, hosts' genomes can have a significant impact on microbiome composition [3,23,29].

While fecal samples are the predominant method from which to study gut microbiomes, differences do exist between microbial composition in the gut and in feces [39]. Even within the gut, the composition in gizzard, upper intestine, and lower intestine are not identical [16]. Since we are interested in directly measuring gut microbial composition, we collected samples from the upper intestinal tract of birds in natural habitat.

Our primary aim for this study was to expand our current catalog of gut microbiome diversity of wild bird species, which is an understudied aspect of bird biology. Secondly, we aimed to examine the microbiota within the gut, rather than feces, to gain insight into their potential effect on the host. For this purpose, we studied the microbiota of three wild bird species collected at the same location during the same season. Each bird species belongs to a different family within the same order, the Passeriformes: Cedar Waxwings (*Bombycilla cedrorum*, Bomycillidae), American Robins (*Turdus migratorius,* Turdidae), and Dark-eyed Juncos (*Junco hyemalis*, Passerellidae).

All three species of birds we tested are known to switch diets during the fall and winter season. American Robins switch from a mostly insectivorous diet to a mostly frugivorous one. Cedar Waxwings will switch from a mostly frugivorous to an almost exclusively frugivorous. Dark-eyed Juncos will switch from an omnivorous diet to a near exclusive granivorous diet [26]. Both Cedar Waxwings and American Robins have been observed to feed on cedar berries (Cedar Waxwings mostly), as well as fruits of crabapple and hawthorn [33,40,42]. Since these birds have different forage patterns, our study also aimed to compare microbial compositions of sympatric, heterospecific species that belong to the same Order during the cold months. To reduce the chance of postmortem change in microbial composition, samples were collected immediately after their death due to accidental collision with windows.

2. MATERIAL AND METHODS

2.1. Samples

Four Cedar Waxwings (samples CW1-4), three American Robins (samples R1-3), and two Dark-eyed Juncos (samples DJ1-2) were collected on the Indiana University Kokomo campus (Kokomo, Indiana USA), except for sample DJ2 which was collected one mile south of campus. The samples were collected as part of daily monitoring for birds that are injured or have died due to window collisions. To minimize the effects of postpartum change in bacterial composition, only recently deceased birds were processed further for DNA extraction. Injured birds were transported immediately to a bird rehabilitation center. Only birds that were collected in October and November of 2019 and 2020 were analyzed. The information and sample names are summarized in the Table 1. After collecting recently deceased birds, they were either immediately processed or placed in the freezer until dissection. Once birds were dissected, the intestines were removed in sections using sterile dissection equipment. The upper intestine was then placed into a sterile container and kept at -80°C until DNA extraction.

Table 1. The summary of sample types and collection dates

Sample ID	Collection date	Common name	Order	Family	Species
CW1	10/8/2020	Cedar	Passeriformes	Bombycillidae	*Bombycilla cedrorum*

CW2	11/10/2020	Waxwings			
CW3	11/16/2020				
CW4	10/7/2020				
R1	10/30/2020	American Robins	Passeriformes	Turdidae	*Turdus migratorius*
R2	11/6/2019				
R3	11/28/2019				
DJ1	10/12/2020	Dark-eyed Juncos	Passeriformes	Passerellidae	*Junco hyemalis*
DJ2	11/25/2019				

2.2. DNA extraction and sequencing

The DNA was extracted from 100 mg of each sample using the E.Z.N.A. Stool DNA kit (Omega-Biotek Inc., GA, USA) according to the manufacturer's instructions. RNA was removed by RNase treatment at 37°C. The extracted DNA was visualized with 1% agarose gel electrophoresis and stained with ethidium bromide solution. The concentration and quality of the DNA was calculated from the measurement of absorbance at 260 nm and 280 nm using the spectrophotometer. A260/A280 between 1.80 and 1.90 were further processed for sequencing. 16S rRNA DNA library of V3-V4 region was constructed and sequenced with Illumina MiSeq at Psamogen Inc. (MD, USA). Between 101,826 and 172,376 raw reads were generated.

2.3. Bioinformatic and statistical analyses

The Illumina Miseq sequence reads were assembled and processed using micca [2]. Reads were trimmed to remove barcode and primer sequences. Low quality reads with average quality scores of less than 25 were discarded. Assembled reads from each sample were classified into operational taxonomic unit (OTU) of 97% sequence similarity. Taxonomic information was assigned to each OTU using the Ribosomal Database Project (RDP) classifier. A single OTU matched a chloroplast-derived sequence. This sequence was removed from further analysis. A phyloseq object was created using phyloseq [27] and further analyzed by R [21]. The reads were rarified to an even depth. The multiple sequence alignment was performed using msa command using the Nearest Alignment Space Termination method [12]. Green gene core set was used a template. The rooted phylogenetic tree was constructed.

The pairwise dissimilarities were calculated using Jaccard distance, Bray-Curtis dissimilarity index, weighted and unweighted UniFrac distances with packages vegan and phyloseq [14,27]. Principal coordinate analysis (PCoA), RDA, and CCA methods were employed to analyze the dissimilarity. Since similar conclusions were drawn from each result, not all of the results are shown. Hierarchical clustering of dissimilarity was calculated using hclust with the Ward's method. Permanova and pairwise Adonis function of vegan was utilized for beta diversity hypothesis testing. PCoA, heatmap and bar plots were constructed using vegan, ggplot2, and ggbiplot in R [38,41].

3. RESULTS

Between 101,826 and 172,376 reads were obtained for each sample by Illumina MiSeq sequencing. The rarefaction curve shows that enough sequencing depth was achieved to capture the diversity within each sample (Fig. 1). The reads were classified into 633 total OTUs. The alpha diversity of each sample was analyzed using the Shannon's diversity index (Fig. 2). There were large variations in diversity index in all species (Fig. 2a). There were no statistically significant differences in diversity between species (Mann Whitney U test, $P = 0.27$ for Cedar Waxwing vs Dark-eyed Junco; $P = 0.4$) for Dark-eyed Junco vs American Robin; and $P = 0.86$) for Cedar Waxwing vs. American Robin). Also, no significant difference was detected for alpha diversity of samples collected in the year 2019, compared to the year 2020 ($P = 0.73$, Fig. 2b). The

collection month (October vs. November) also did not impact the alpha diversity (P = 0.29).

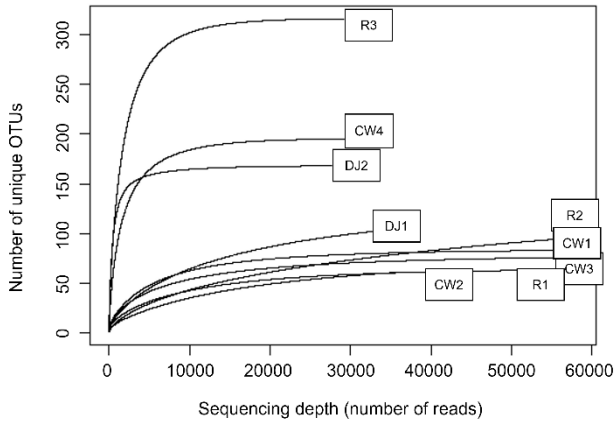

Fig. 1. Rarefaction curve of the Illumina reads.
Sample IDs are listed in the table 1.

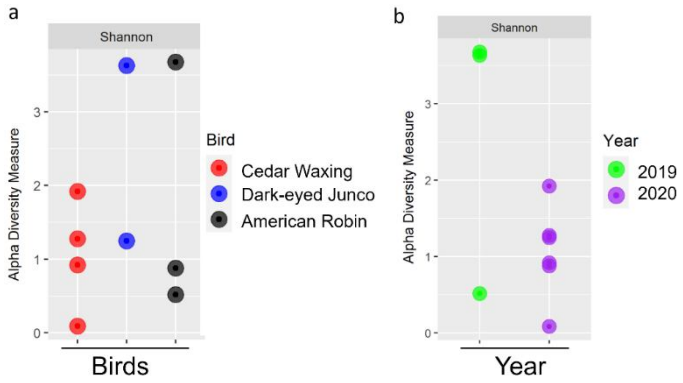

Fig. 2. Comparison of alpha diversity index.
The Shannon diversity index of OUTs in each sample is grouped according to the (a) bird species and (b) sample collection year. There were no differences between the groups with statistical significance.

The beta diversity of the samples was then compared using the principal coordinate plot (Fig. 3). The principal coordinate plot, displaying the weighted and unweighted UniFrac distance, does not show distinct clustering of samples based on taxonomic group (species) nor based on the collection year or month (Fig. 3).

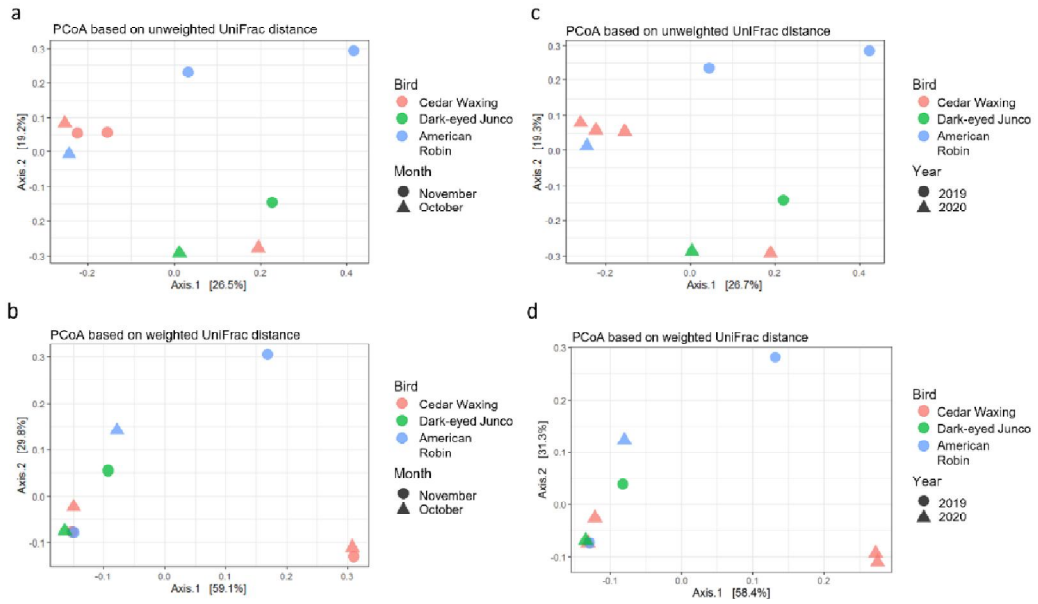

Fig. 3. Principal coordinate analysis of beta diversity based on weighted or unweighted UniFrac distance.
Unweighted (a and c) or weighed (b and d) UniFrac distance methods were used. The samples are colored according to the species (pink: Cedar Waxwings, green: Dark-eyed Juncos, and blue: American Robins). The shape represents the month (a and b) or year (c and d) of sample collection date.

Each sample contained 60 to 315 unique OTUs. The taxonomy was assigned for each OTU. In four samples (CW1, CW2, R1, and R2), a single OTU composed a significant portion of the microbiota (74%, 98%, 71%, and 84% of total sequences, respectively). The most abundant OTU in a Cedar Waxwing sample (CW2) and an American Robin sample (R1) belong to the genus *Escherichia/Shigella* (OTU1). The most abundant OTU of the sample CW1 is *Helicobacter* (OTU2). Another *Helicobacter* sequence (OTU5) was also abundant in other Cedar Waxwing samples: in CW1 (11%) and CW3 (29%). *Yersinia* sequence (OTU3) was only abundantly present in sample R2 (57%). The second most abundant OTU (OTU10), which comprised 26% of the sample R2, was also *Yersinia* sequence.

There are many other proteobacterial sequences that were prevalently present, including *Campylobacter* (OTU4), Enterobacteriaceae (OTU6 and OTU11), and *Aeromonas* (OTU7). The phyla Firmicutes, Tenericutes, Deinococcus-Thermus and Actinobacteria were also found in multiple samples at a high abundance. The taxonomy and relative abundance of OTU in each sample are visualized in Figure 4. The most abundant bacterial phyla were Proteobacteria (colored brown in the Fig. 4a) in all samples. Firmicutes (colored light green) were also highly represented in samples CW4, DJ1, and DJ2, and R3, respectively.

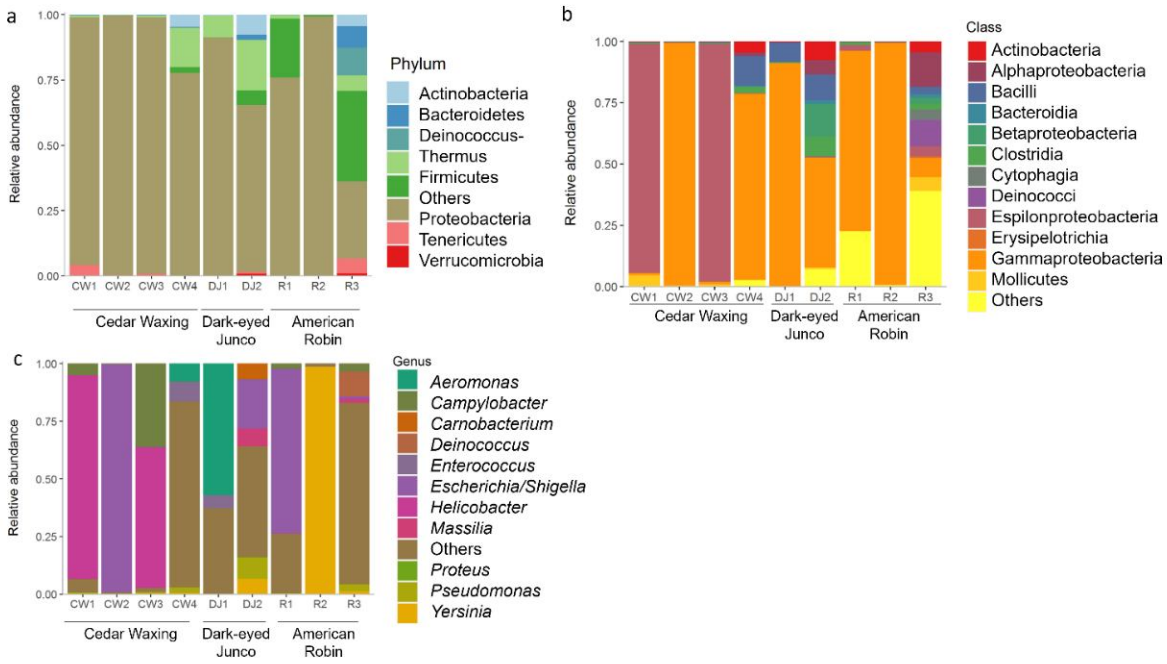

Fig. 4. The relative abundance of OTUs classified into unique taxonomic groups.

Taxonomic classification at (a) phylum, (b) class, and (c) genus is shown. The taxonomic units that represent greater than 1% abundance are included. The sample IDs are summarized in the Table 1. Abbreviations: CW: Cedar Waxing; DJ: Dark-eyed Junco; and AR: American Robin.

Despite proteobacteria being uniformly the most abundant bacterial phyla, different classes of proteobacteria were abundant in each sample (Fig. 4b). In CW1 and CW3, epsilonproteobacteria was the most abundant class. As discussed above, the most abundant sequences in these two samples were that of genus *Helicobacter* (OTU2 and 5) and *Campylobacter* (OTU4), which both belong to epsilonproteobacteria (Fig. 4c). In other samples, gammaproteobacterial sequences were abundant (Fig. 4b). *Escherichia/Shigella* (OTU1) and *Yersinia* (OTU3 and 10) belong to the class gammaproteobacteria (Fig. 4c). Additionally, the most abundant OTU in CW4 (OTU6) was a gammaproteobacterial sequence with no known homolog at the genus level. Figure 5a summarizes the fraction of each OTU present in each sample. Samples CW4, R3, and DJ2 contained many unique OTUs that were not present in others. Other samples contained relatively smaller numbers of OTUs, but these OTUs are present in most samples.

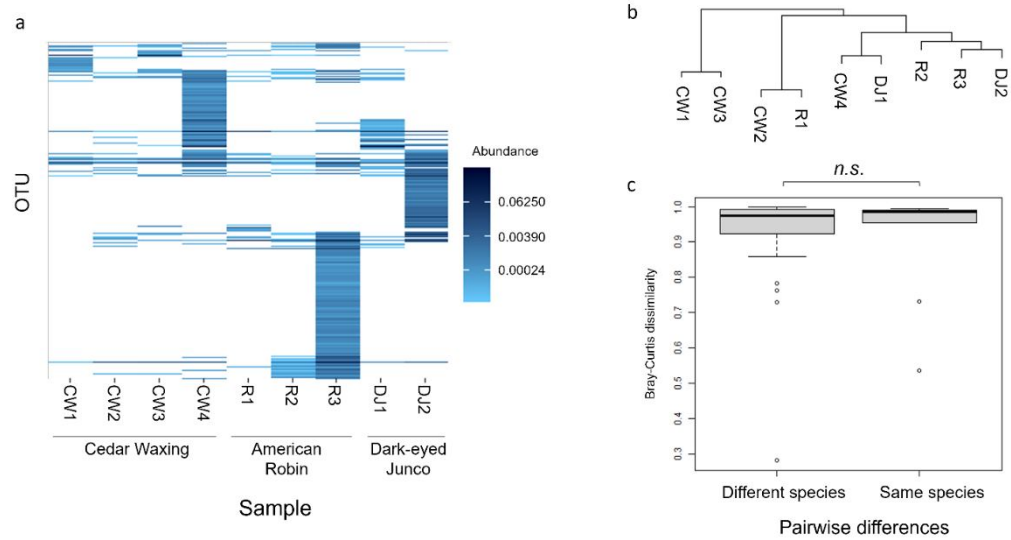

Fig. 5. The heatmap and cluster dendrogram of OTUs in each sample.

(a) Heatmap of the OTU with the abundances greater than 10-5 are shown. (b) Ward's hierarchical agglomerative clustering method was applied to the Bray-Curtis distance between samples. Abbreviations: CW: Cedar Waxing; DJ: Dark-eyed Junco; and AR: American Robin. (c) Pair-wise comparison of Bray-Curtis dissimilarity index. The dissimilarity of microbiota of samples belonging to the same species (right) and dissimilarity between samples belonging to different species (left) are shown.

Pairwise comparison of Bray-Curtis dissimilarity was measured. The dendrogram of Bray-Curtis distance shows clustering of 1) CW1 and CW3, 2) CW2 and R1, 3) CW4 and DJ1, and 4) R3 and DJ2 (Fig. 5b). An intraspecies pair (CW1 and CW3) has a similar microbiota comparison. However, there were certain pair of samples with low dissimilarity (e.g. CW2 and R1; CW4 and DJ1). We then pooled a pairwise comparison of Bray-Curtis dissimilarity into 1) between the intra-species and 2) inter-species samples (Fig. 5c). This comparison did not show a statistically significant difference (Mann Whitney U test, $P = 0.87$) (Fig. 5c). A pair of microbiota from different species (R1 and CW2) had low dissimilarity index of 0.282. This is lower than the smallest dissimilarity index when two of the CW samples were compared (0.537 for CW1 and CW3). Pairwise comparison based on collection year or month also did not show a significant difference ($P = 0.48$ and $P = 0.30$, respectively).

Figure 6 displays the number of OTUs that are unique to each species as well as commonly found in two or three bird species. The values represent the number of unique OTUs that were found in at least one sample in each species. Out of 632 total OTUs found in this study, 73 OTUs (12% of total) were detected in all three species. Dark-eyed Juncos, Cedar Waxwings, and American Robins carried 92, 129, and 242 unique OTUs that were not found in other species, respectively.

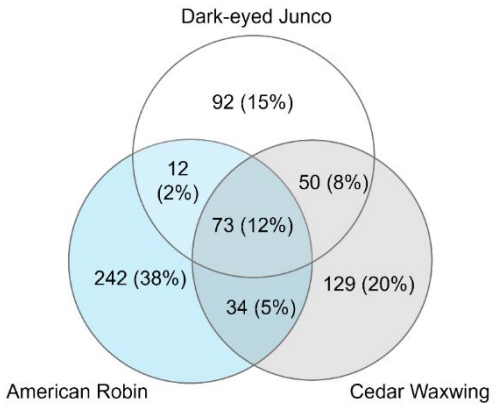

Fig. 6. The Venn diagram of unique OTUs observed in each species.

The numbers of OTUs that were present in at least one sample from each species were counted.

4. DISCUSSION

In this study, we discovered that three sympatric Passeriformes species in the wild exhibited high intraspecies variation of microbiota composition in the upper intestinal tract. While uniformly the most abundant phylum was proteobacteria, a high dissimilarity of composition at the genus and class level was observed. Because we relied on our sample collection from birds that accidentally died due to collision with windows, the numbers of our sample were limited. In particular, we were able to collect only two Dark-eyed Junko samples, limiting our ability to draw conclusions with statistical significance. However, our approach allows an ethical means to gain samples from the intestinal tract of the birds in their natural habitats and study their microbiota.

Our results match a trend of weaker association between host's phylogeny and microbiota variation in birds that have been observed previously [36,44]. The striking similarity between birds of different species suggests the possibility that interspecies exchange of gut microbes happens among sympatric birds. Further investigation to search for the cause of observed high intraspecies dissimilarity, as well as interspecies similarity, such as examining the birds' individual migratory history, food content in the gut, and sex, is needed.

Flighted birds and bat microbiota generally have fewer bacteria responsible for food fermentation, while the number of proteobacteria is higher. A relatively high amount of proteobacteria have been observed in feces of Passeriform species [19]. In wild neotropical birds, the proportion of proteobacteria is higher in bird consuming fruits than in birds consuming grains [16]. Our study also detected a high proportion of proteobacteria in all samples except for a relatively smaller proportion in a robin sample (R3 in Fig. 4). Whether or not the difference between R3 and other samples reflects the types of diet immediately before sampling requires further investigation.

The most common OTUs belonged to the genus *Escherichia/Shigella, Helicobacter, Yersinia, Campylobacter*, unknown genus of Enterobacteriaceae, and *Aeromonas*. Although our study does not allow identification of specific function, some of the members of these genera are known pathogens [24]. The presence of human pathogens in bird feces has been described [8]. The roles of proteobacteria in the gut of flighted animals are of interest not only for understanding their diet but also for epidemiological concerns.

The postmortem change in gut microbial composition has been studied in humans [18]. Increases in *Bacteroides* and *Lactobacillus* have been detected, but changes were minimum within the first 24 hours. No significant increase in the abundance of proteobacteria was observed. It remains unknown whether the same sets of bacterial species will change abundance in birds postmortem as in humans. Since our samples were relatively fresh (from a few minutes up until a few hours), the postmortem change should be low. However, a future study to determine the time-dependent change postmortem of microbial composition in bird species will be needed.

5. CONCLUSION

In conclusion, our study has shown that birds of Passeriformes in natural habitats carry highly diverse sets of gut bacteria. This suggests the significant effects of diet and/or other environmental factors on their microbiome. Due to the presence of bacterial strains with known associations with pathogenicity in humans, this is an area that deserves further attention and studies.

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