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HOST MICROBE INTERACTIONS

Comprehensive Molecular Characterization of Bacterial Communities in Feces of Pet Birds Using 16S Marker Sequencing

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Abstract Birds and other animals live and evolve in close contact with millions of microorganisms (microbiota). While the avian microbiota has been well characterized in domestic poultry, the microbiota of other bird species has been less investigated. The aim of this study was to describe the fecal bacterial communities of pet birds. Pooled fecal samples from 22 flocks representing over 150 individual birds of three different species (*Melopsittacus undulatus* or budgerigars, *Nymphicus hollandicus* or cockatiels, and *Serinus canaria* or domestic canaries) were used for analysis using the 16S rRNA gene sequencing in the MiSeq platform (Illumina). Firmicutes was the most abundant phylum (median 88.4 %; range 12.9–98.4 %) followed by other low-abundant phyla such as Proteobacteria (median 2.3 %; 0.1–85.3 %) and Actinobacteria (median 1.7 %; 0–18.3 %). Lactobacillaceae (mostly *Lactobacillus* spp.) was the most abundant family (median 78.1 %; 1.4–97.5 %), especially in budgerigars and canaries, and it deserves attention because of the ascribed beneficial properties of lactic acid bacteria. Importantly, feces from birds contain intestinal, urinary, and reproductive-associated microbiota thus posing a serious problem to study one anatomical region at a time. Other groups of interest include the family Clostridiaceae that showed very low abundance (overall median <0.1 %) with the exception of two samples from cockatiels (14 and 45.9 %) and one sample from budgerigars (19.9 %). Analysis of UniFrac metrics showed that overall, the microbial communities from the 22 flocks tended to cluster together for each bird species, meaning each species shed distinctive bacterial communities in feces. This descriptive analysis provides insight into the fecal microbiota of pet birds.

Keywords Microbiota · Pet birds · 16S rRNA gene · PICRUSt · Health

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Introduction

Animal bodies, especially the digestive, urinary, respiratory, and reproductive tracts, are permanently colonized by millions of phylogenetically and metabolically diverse microscopic life forms (microbiota) [1]. These microorganisms are vital for many key biological processes in their hosts such as immune processes and digestive pathways that are necessary for maintaining health and well-being [2, 3]. This intimate microbiota-host relationship is the result of the synchronized evolution of both life forms over millions of years, a complex process known as coevolution [4, 5] where microorganisms establish communities accordingly to host characteristics such as diet and gut type [6] in close communication with the immune system [7].

There are about 10,000 species and 22,000 subspecies of birds described to date [8]. Throughout its evolution, each bird group has developed unique anatomical, physiological, behavioral, dietary, and social traits that, expectably, have a strong genetic component from the host [9]. These characteristics also make birds some of the most interesting organisms to study host-associated microorganisms because high interspecies host diversity likely helps originate favorable conditions for a wide range of diverse microbial adaptations.

While the relationship of microorganisms to health and physiology is well documented in humans and some laboratory animals [2], we know much less about the role of microbial communities in other animal species including birds. The avian gut microbiota (especially fecal) has been characterized in several studies especially from domestic chickens [10–23] while other non-domestic birds have arguably received less attention [24–29]. The reasons for this lack of representativeness are various but may relate to the use of some bird species (e.g., chicken) as a food source and our increasing desire to intensify food production while maintaining animal health. Also, the collection of biological samples from several groups of birds is a difficult task because most birds are small in size, difficult to catch, and/or occupy rare niches in remote geographical localizations. Moreover, some birds get very anxious when handled by people, a phenomenon of unknown consequences for the analysis of their associated microbial communities.

The avian microbiota is of interest because of the complex host-microbiota relationship and its consequences for the evolution of both life forms. The avian microbiota is also of interest for the medical community (both human and veterinary) because birds may harbor organisms with pathogenic potential [29–34]. All around the world, millions of pet birds from different species live in close contact with people and owners are often exposed to their droppings and other body secretions, which have shown to contain potential pathogenic organisms such as several strains of *Clostridium* and *Campylobacter* spp. [29–34]. While this phenomenon is important for both veterinary and human medicine, pathogen identification has received more attention in poultry [32] compared to other avian species, including pet birds. Pathogen identification in pet birds is also relevant because people often handle the trays of their birds' cages by hand without the use of personal protective equipment (e.g. protective clothing and gloves).

In an effort to describe the bacterial composition shed in feces by pet birds, and also to promote the study and understanding of microbial coevolution with animal hosts, the objective of the present study was to characterize the fecal microbiota of bird species commonly kept as pets. For this end, we used high-throughput sequencing of a semiconserved region of the 16S rRNA gene from fecal material of three bird species. The results may inform future studies on microbial ecology as well as animal and human health.

Methods

Sample Collection

This research involved collection of fecal samples only and all applicable international, national, and/or institutional guidelines for the care and use of animals were followed. Fresh fecal samples were collected from birds belonging to private owners and local businesses in Monterrey and its metropolitan area in northeast Mexico. We sampled feces from three different bird species (*Melopsittacus undulatus* or budgerigars, *Nymphicus hollandicus* or cockatiels, and *Serinus canaria* or domestic canaries) because these are the most common pet birds in our area. Fecal sampling from all the birds was performed during the same period of time thus minimizing potential effects of weather and other factors on the birds' behavior and physiology and consequently on gut physiology and microbiology.

Samples were obtained from cages containing birds from the same species but with different numbers of individual birds (2–33), and therefore, each sample represents an aggregate of feces per flock. We chose this method of fecal collection because people often have more than one bird per cage and we did not want to separate individual birds because it could alter the composition of the fecal microbiome (e.g., some birds get very nervous when they are handled by people). We removed the metallic tray on the bottom of the cage, cleaned it, placed new clean paper on it, and waited for the animals to defecate. Collection of feces was performed using a clean sterile spatula and took 20–60 min until one 2-ml plastic tube was filled with enough feces for DNA extraction (~500 mg). Feces were transported to our laboratory and frozen at −20 °C until processing. Table 1 shows useful information about the birds from which the fecal samples were obtained. All individual samples were collected from independent non-relative birds with the exception of two samples that were collected from relative birds (i.e., parents and their newborn chicks); therefore, we treated each sample individually (i.e., no attempt to cluster samples in groups was done).

DNA Extraction

Total genomic DNA was obtained from 100 mg of all fecal samples using bead-beating followed by DNA isolation and purification using a commercial kit (Wizard Genomic DNA Purification Kit). This kit yielded more DNA (>40 μg/μL) than did column-based kits used in previous studies [35] thus potentially providing a wider range of potential targets for PCR and sequencing.

16S Sequencing

Purified DNA samples were processed at Molecular Research LP (MR DNA, Shallowater, Texas, USA). DNA samples were

Table 1 Information about the birds from whom the fecal samples were obtained from

Sample ID	Bird species	Number of birds	Feeding	Ownership
MU1	*Melopsitaccus undulatus* (budgerigars)	9	Canary grass only	Private
MU2	*Melopsitaccus undulatus* (budgerigars)	7	Canary grass only	Private
MU3	*Melopsitaccus undulatus* (budgerigars)	10	Canary grass only	Private
MU4	*Melopsitaccus undulatus* (budgerigars)	5	Canary grass only	Private
MU5	*Melopsitaccus undulatus* (budgerigars)	3	Canary grass and fecal matter, parents feeding	Private
MU6	*Melopsitaccus undulatus* (budgerigars)	3	Canary grass only	Private
MU7	*Melopsitaccus undulatus* (budgerigars)	3	Canary grass and fresh fruits	Private
MU8	*Melopsitaccus undulatus* (budgerigars)	6	Mix of seeds	Private
MU9	*Melopsitaccus undulatus* (budgerigars)	6	Canary grass only	Private (same as sample MU5)
MU10	*Melopsitaccus undulatus* (budgerigars)	8	Mix of seeds	Commercial
MU11	*Melopsitaccus undulatus* (budgerigars)	33	Canary grass only	Commercial
MU12	*Melopsitaccus undulatus* (budgerigars)	7	Mix of seeds	Private
SD13	*Serinus canaria* (island canary)	14	Canary grass, fruits, vegetables, boiled eggs	Private
SD14	*Serinus canaria* (island canary)	5	Mix of seeds	Private
SD15	*Serinus canaria* (island canary)	6	Mix of seeds, lettuce and broccoli	Private
SD16	*Serinus canaria* (island canary)	7	Mix of seeds, fruits and vegetables	Commercial
SD17	*Serinus canaria* (island canary)	15	Mix of seeds	Commercial
SD18	*Serinus canaria* (island canary)	8	Mix of seeds	Commercial
NH19	*Nymphicus hollandicus* (cockatiels)	6	Girasol seeds, fruits and vegetables	Private
NH20	*Nymphicus hollandicus* (cockatiels)	2	Girasol and mix of seeds	Commercial
NH21	*Nymphicus hollandicus* (cockatiels)	3	Girasol and mix of seeds	Commercial
NH22	*Nymphicus hollandicus* (cockatiels)	5	Girasol and mix of seeds	Commercial

used for PCR amplification of the semiconserved V4 region of the 16S rRNA gene using primers GTGYCAGCMG CCGCGGTAA and GGACTACNVGGGTWTCTAAT [36]. Pools of PCR products were compiled and verified in agarose gels before sequencing using the Illumina MiSeq platform as described elsewhere [36–38].

Bioinformatics

Raw sequencing files were shared by MR DNA through BaseSpace (Illumina) and comprised two FASTQ files, one each for the forward and reverse sequence reads. Quality filtering and downstream analysis was performed using both read files separately as well as the file containing the joined read files (full file). Unless stated otherwise, default parameters and scripts in QIIME [39] v. 1.8 were used for all analysis using the QIIME Virtual Box. Raw reads (original FASTQ files) were joined using the QIIME script join_paired _ends.py. All sequence data have been submitted to the Sequence Read Archive (SRA) and are freely available at the NCBI (BioProject: PRJNA301166).

Operational taxonomic units (OTUs) were assigned using two different approaches. First, using UCLUST v.1.2.22 [40] as implemented in QIIME using the open-reference clustering algorithm described in [41] for OTU description, alpha and beta diversity analyses. In our experience, this method to pick OTUs is very useful to catalog sequences into groups especially when using samples from poorly described environments (e.g., feces of some bird species). Second, using the pick_closed_reference_otus.py QIIME script as required for further analysis using Phylogenetic Investigation of Microbial Communities by Reconstruction of Unobserved States (PICRUSt, [42]) for predicting the functional profile. For both of these two approaches (closed- and open-reference OTU picking), we used the GreenGenes v.13.5 16S database. Possible chimeras were removed using ChimeraSlayer in QIIME after OTU assignments. All Cyanobacteria, Streptophyta, and mitochondrial reads were removed as these are likely derived from plant and animal material.

Species Identification

As part of the sequencing service provided by MR DNA, the raw FASTQ files are accompanied by a taxonomic classification of OTUs using not only the commonly used GreenGenes 16S database but also other 16S databases such as the Ribosomal Database Project and NCBI. The use of these other databases greatly improves the assignment of OTUs to

specific taxa and is very useful to obtain a first insight of OTU taxonomy especially at the genus and species level. We used the taxonomy assignments provided by MR DNA to look for potential species of interest and then used our 16S sequences to confirm the results using BLAST against a 16S database from the NCBI (see "Species Identification" section in the "Results" section).

Statistics

With the exception of singletons (OTUs represented by one single sequence), in this study, we did not discard any OTU (using for example filter_otus_from_otu_table.py in QIIME to remove OTUs with low number of sequences) because doing so removes potentially useful information from the bacterial communities being studied and also because of the ambiguity associated with sequence similarity [43, 44] and grouping of sequences into OTUs [45–47]. This is especially important considering the fact that OTUs are rarely monophyletic [48] and the little we know about 16S molecular evolution [49, 50]. Alpha diversity was estimated using the number of OTUs (species richness), the Shannon [51] and PD whole tree [52] diversity indexes, and the Chao1 [53] metric. These metrics are useful for estimating microbial diversity and richness (as shown by many studies) but are not exempt of disadvantages (see [54]). In order to obtain a better insight into the clustering of bacterial communities, both weighted (takes into account changes in relative taxon abundance) and unweighted (does not consider information about taxon abundance) UniFrac metrics were used for beta diversity [55]. The non-parametric ANOSIM and Adonis tests were used to determine differences in microbial communities in QIIME. Principal coordinate analysis (PCoA) was used to explore similarities among microbial communities using UniFrac distance metrics in PAST [56]. PICRUSt [42] and STAMP [57] were used to predict and visualize the functional profiling of the fecal microbiota, respectively. The weighted nearest sequenced taxon index (weighted NSTI) scores were calculated to assess the accuracy of the PICRUSt predictions. In this descriptive analysis, we did not attempt any statistical comparison of the abundance of the different taxa because pooling fecal samples from different birds erases all interindividual variability within each flock, and each pooled fecal sample was obtained from varying numbers of birds and the different numbers of samples collected for the analysis from each bird species.

Results

Sample Collection

Pooled fecal samples were successfully collected and processed from 22 flocks representing 100 budgerigars (12 samples total), 55 canaries (6 samples), and 16 cockatiels (4 samples) (Table 1).

Sequencing Results

As expected (see [36]), the analysis of forward, reverse, and assembled 16S reads yielded similar results with respect to taxonomic classification and alpha and beta diversity. In this current study, this is explained by the fact that the primers used to amplify the V4 region span a 300-bp region; therefore, all sequences (forward, reverse, and joined reads) represent basically the same region. For the sake of space and clarity, only those results obtained from the joined reads are presented and discussed (1.7 million joined reads after quality filtering and open-reference OTU picking; median length 300 bp).

Bacterial Taxonomic Composition

A total of 13 different bacterial phyla were identified but Firmicutes alone accounted for almost 90 % of all the 16S reads (median 88.4 %; range 12.9–98.4 %). The second most abundant phylum was Proteobacteria (median 2.3 %; range 0.1–85.3 %), followed by Actinobacteria (median 1.7 %; range 0–18.3 %) and other low abundant groups. The phylum Bacteroidetes, which is often found in high abundance and plays an important role in the mammalian gut, was found to be very low in all of our 22 samples (median 0.03 %; range 0–1.7 %).

Most bacterial phyla are composed by many different groups; therefore, it is more informative to look at deeper taxonomic levels. Over 100 different bacterial groups were detected at the family level, but only 25 of them accounted for over 95 % of all the groups, with wide variation among individual samples, especially among bird species (Fig. 1). For example, Lactobacillaceae (mostly *Lactobacillus*) was the most abundant family (median 78.1 %) but showed high variability among samples ranging from ~1 % to almost 100 %. High proportions of Lactobacillaceae were found mostly in canaries and budgerigars (Fig. 1). Pseudomonadaceae (mostly *Pseudomonas*, class Gammaproteobacteria) showed very low abundance (overall median: 0.7 %) with the exception of two samples from canaries (17.0 and 45.0 %). Clostridiaceae was also very low (median among all samples <0.1 %) with the exception of two samples from cockatiels (14 and 45.9 %) and one sample from budgerigars (19.9 %). Other groups that exemplify this high variability among bird species include Lachnospiraceae (three out of the four samples from cockatiels had >28 % while all the other samples had <0.8 %) and Erysipelotrichaceae (three out of the four samples from cockatiels had >10 % while all the other samples had <0.5 %) (Fig. 1). Overall, the samples tended to cluster

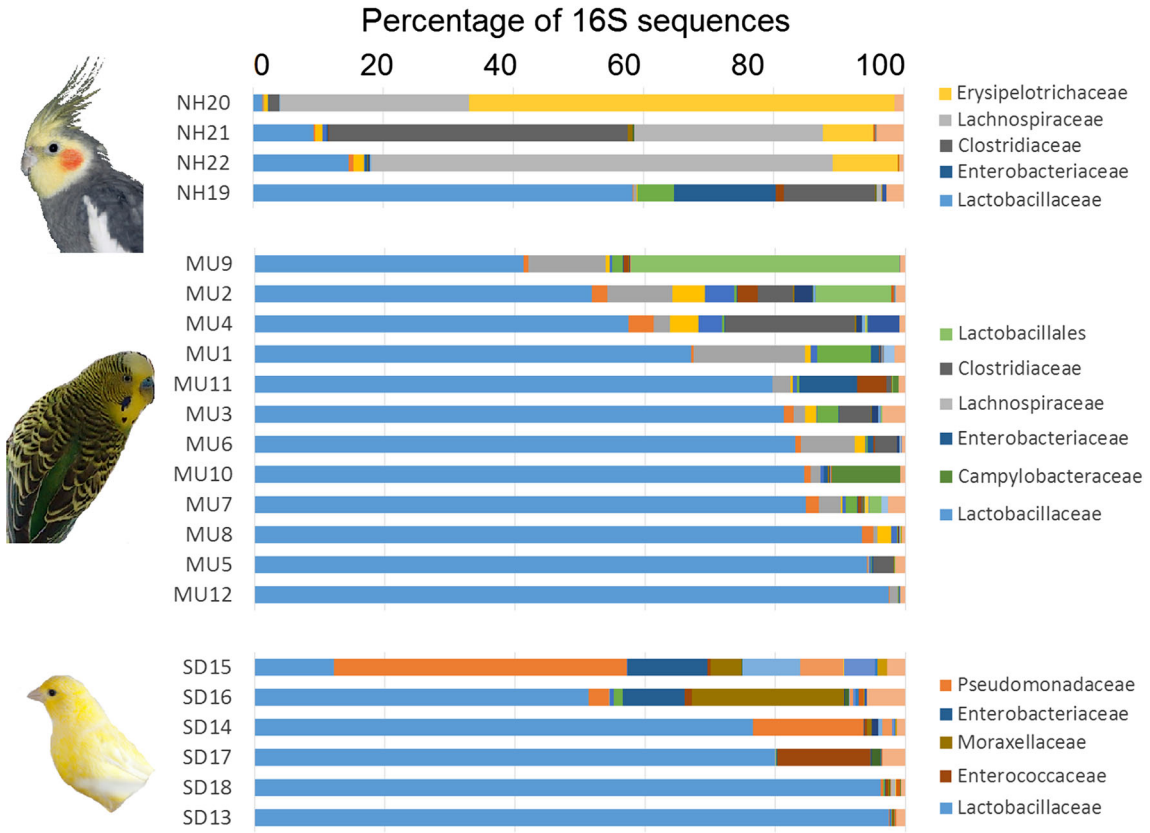

Fig. 1 *Bar charts* showing percentage 16S sequence reads at the family level. Bars are organized from the lowest to the highest abundance of Lactobacillaceae for each bird species (MU *Melopsittacus undulatus* or budgerigars, SD *Serinus canaria* or domestic canaries, NH *Nymphicus hollandicus* or cockatiels). For the sake of clarity, only the numerically abundant groups are mentioned in the text. All the rest of the groups are *colored* in the charts but please note that they were only included to aid the visualization of the diversity of all the other many taxa (a total of 25 families comprised over 95 % of all groups in all 22 samples, see main text for details)

together for each bird species when using percentages of 16S sequences from the most abundant families (Fig. 2).

Species Identification

Often, the 16S rRNA gene sequence similarity is not a good indicator of either true genomic phylogenetic relationship [59] or metabolic profile [60]. In groups like *Escherichia coli*, it is simply impossible to differentiate it from *Shigella* based on the 16S rRNA gene. These facts must be considered especially when using small fragments of the 16S gene to relate sequences to specific genus or species. Other factors affecting the fitness of an organism during health or disease exert caution when finding similarities among 16S gene sequences and drawing relationships. All things considered, in this study, we generated 16S sequences closely matching 16S sequences from *Clostridium disporicum* and *C. colinum*, both potential pathogens for human and animal health [30, 34, 61] (see Supplementary information for more information about these sequences). Interestingly, these sequences were only found in samples from cockatiels and in this study, all cockatiels were clinically healthy.

Alpha Diversity

Alpha diversity indices are shown in Table 2. Although no statistical test was performed to compare our samples, each index can shed light into the fecal bacterial diversity of pet birds. For example, the Shannon diversity index [51] gives more weight to the numerically dominant OTUs and expectably, budgerigars were associated with the highest Shannon indexes (Table 2). On the other hand, the nonparametric Chao1 index, which is known to be an accurate estimator of richness, was highest in cockatiels and lowest in canaries (Table 2).

All sequences were assigned to a total of 8751 distinctive OTUs at 97 % similarity. Using a rarefaction depth of 23,000 sequences per sample (min 23,224; max 220,000 sequences per sample after quality filtering, OTU assignments, and removal of plant and animal-derived sequences), the number of OTUs did not reach a plateau in rarefaction analysis especially in two samples from canaries, meaning more sequences are needed to fully estimate the bacterial OTU diversity in the feces of these birds (Supplementary Fig. S1). Chao1 indexes also did not level out in those two samples from canaries compared to the

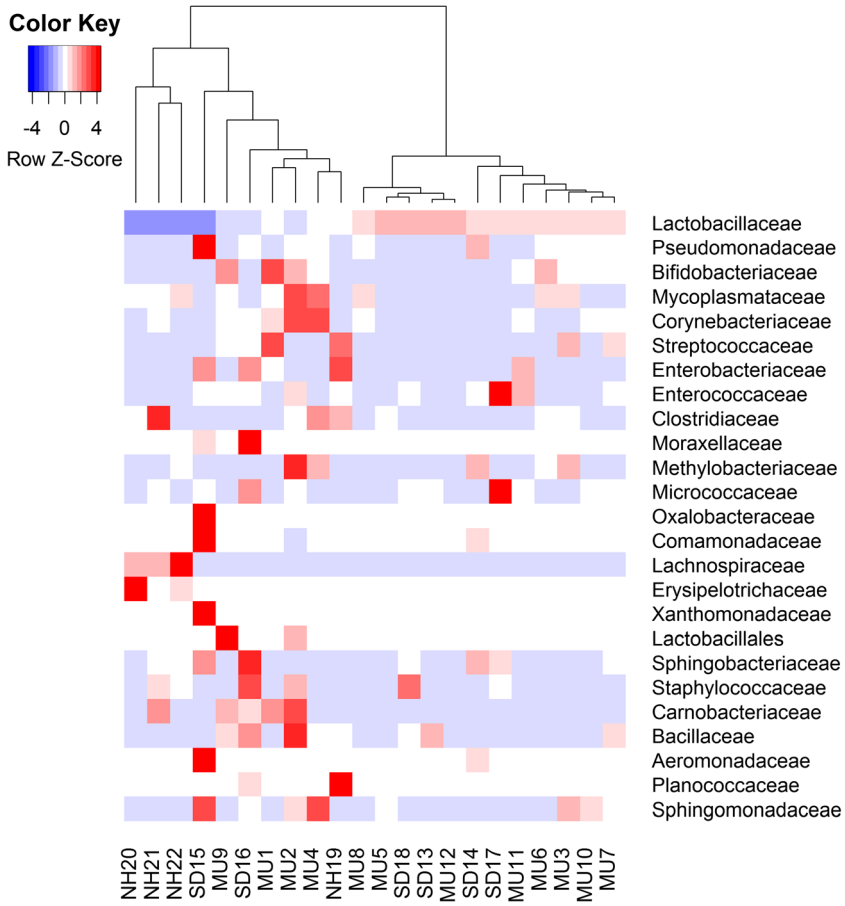

Fig. 2 *Heat map* showing the clustering of relative abundances of the 25 most bacterial groups at the family level. The heat map was created using the function heatmap.2 in the library gplots in R v. 3.2.1 [58]

rest of the samples (Fig. S1). It must be emphasized that these calculations used a relatively new OTU picking algorithm that do not discard sequences immediately when not found in the reference sequence dataset [41]. Other reasons for this high number of OTUs may include the fact that each sample is a pool of fecal samples from several birds, which may increase the diversity of the sequences; however, pooled samples were also collected from other canaries, budgerigars, and cockatiels and these other samples did not show this high amount of richness. It is also possible that the generated sequences were very diverse among them (as to generate multiple new groups of OTUs) and/or do not have close representatives in current 16S databases, as this is the first massive report of bacterial 16S rRNA genes in the pet birds studied. Canaries in this study were fed with a more varied diet (Table 1), which could have also contributed to the observed OTU diversity. Finally, canaries have been extensively modified for at least four centuries to reach certain phenotypes and people (especially birds' dealers) often cross animals with different characteristics, a pressure that may have propitiated higher microbial diversity. These reasons for explaining the high number of OTUs in those two samples from canaries are only speculations that need further investigation.

Beta Diversity

We used both weighted and unweighted UniFrac metrics because they can lead to different insights into the association among microbial communities as shown by others [55] including our research group [37, 38]. Regardless of the metric used, PCoA revealed that overall, cockatiels and canaries

Table 2 Median (minimum-maximum) alpha diversity indices

	Budgerigars	Canaries	Cockatiels	Overall statistics
PD whole tree	53.7 (42–96.1)	52.4 (24.8–109.8)	59.7 (29.6–87.8)	53.7 (24.8–109.8)
Chao1	952.3 (747.4–1722.7)	936 (406.5–2283.7)	969.6 (361.8–1588.2)	952.3 (361.8–2283.7)
OTUs	648 (459–1466)	724 (195–2136)	782 (290–1374)	648 (195–2136)
Shannon	4.4 (3.3–5.3)	2.1 (1.4–6)	3.2 (1.6–4.6)	4.2 (1.4–6)

tended to cluster separately from each other and from the budgerigar samples (supported by ANOSIM and Adonis test, $P < 0.001$, Fig. 3), strongly suggesting that each bird species shed distinctive microbial populations in their feces. However, weighted UniFrac suggested a much closer association (i.e., less variation) among all the 12 samples from budgerigars and a high variability of canary samples along the axis with the highest variation explained (ANOSIM R value 0.53) while unweighted UniFrac showed a clearer separation of communities (ANOSIM R value 0.81) (Fig. 3). Interestingly, one out of four samples from cockatiels (sample NH19) did not cluster with the rest of cockatiels and remained with budgerigar samples using both weighted and unweighted metrics (note that this sample harbored proportions of Lactobacillaceae that resembled the average Lactobacillaceae in the samples from budgerigars; Fig. 1). This sample was obtained from privately owned birds that were fed with vegetables and fruits, as opposed to the other samples from cockatiels that were collected from commercial businesses and had a less varied diet (Table 1). These results suggest that non-genetic environmental factors (e.g., diet) are also implicated in shaping bacterial communities in birds.

Predicted metabolic Profile of Fecal Microbiota

PICRUSt [42] revealed that the fecal microbiota of budgerigars, cockatiels, and canaries possess a potential complex metabolically rich profile, including protein, carbohydrate, vitamin and lipid metabolism, degradation of natural compounds in plants, bacterial toxins, sporulation, and tetracycline biosynthesis, among others (Supplementary Table S1). Importantly, NSTI scores were low (~0.03) and actually lower compared to PICRUSt predictions from laboratory mice [38], suggesting that the PICRUSt predictions in this current study were accurate. The absence or presence of metabolic features predicted by PICRUSt is worth emphasis because multiple features show no abundance in this and in other studies [62, 63], including some from our research group [37, 38], suggesting that the presence and relative abundance of the predicted traits may be biologically meaningful. Principal component analysis (PCA) showed that these metabolic profiles clustered separately for each bird species, thus supporting the results of beta diversity (especially weighted UniFrac) observed for their respective communities (Supplementary Fig. S2).

Discussion

Birds live and evolve in close contact with complex microbial communities. Thus, modern birds represent a complex biochemical blend of microbial and eukaryotic cells that resulted from thousands of years of slow but constant adaptation. Many studies have been published about the avian microbiota but much more emphasis has been placed on chickens and other domestic poultry. This study described the bacterial composition in the feces of birds that are commonly kept as pets.

Firmicutes was found to be the most abundant phylum followed by other less abundant groups. Interestingly, over half of our samples had >50 % of Lactobacillaceae, suggesting that this group is an important member in the birds studied, especially in budgerigars and canaries. Members from this group (e.g., *Lactobacillus* spp.) may be potentially isolated and used as a species-specific probiotic formulation for pet birds, a topic of great interest in domestic chickens [64]. Other interesting finding from our study is the very low proportion of Bacteroidetes, an important group for gut health in mammals that has been associated with energy harvest and obesity in humans, and the isolated presence of high amounts of Pseudomonadaceae in two samples from canaries only. While recent studies of the avian microbiota have also found low Bacteroidetes (e.g., 7–11 % of all sequences in fecal microbiota of chickens [15, 23] compared to <0.1 % in this current study), proportions of Bacteroidetes in feces of birds varies widely depending on the specific species [65]. This low proportion of Bacteroidetes in this current study is interesting because in humans, two of its most important and abundant members (*Bacteroides* and *Prevotella*) have been associated with diets rich in protein and animal fat and carbohydrates

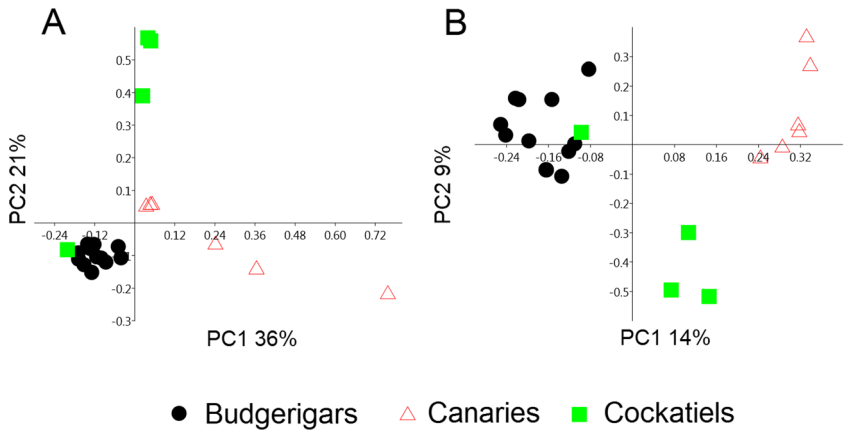

Fig. 3 Principal coordinate analysis (PCoA) plots using weighted (**a**) and unweighted (**b**) UniFrac distance metrics. The plots were created in PAST [56] using the UniFrac distance matrices obtained from QIIME [39]

[66], respectively. Regardless, it is unclear what the bacterial group Bacteroidetes truly represents [67], an important issue that to our knowledge has not been discussed in the animal or avian literature. On the other hand, *Pseudomonas* is one of the most versatile bacterial groups on Earth [68] and this study suggests that this group is an abundant member of the fecal microbiota in at least some canaries.

The UniFrac method was developed by Lozupone and Knight [69] and originally focused on the phylogenetic relationships among microbial communities only (unweighted UniFrac). Roughly, this means that a separation of communities (using for example PCoA) based on UniFrac distances would simply imply that the communities were phylogenetically divergent (i.e., it did not matter whether there was even or uneven numbers of OTUs in the data, a common feature of most massive characterizations of microbial communities). Later on, the same authors introduced a version of UniFrac that could also take into account the number of OTUs present in the samples [55], an important matter in microbial ecology. The use of both weighted and unweighted UniFrac metrics is very useful to understand associations of microbial communities, as shown by our research group and others [37, 38, 70–72]. Although it has been more than 10 years since Lozupone et al. [55] introduced the weighted UniFrac and nicely showed its utility to better understand the similarities among microbial communities, most studies report either the weighted or the unweighted results but not both. In this study, both metrics suggested that each bird species harbors different microbial communities but adding the number of OTUs (weighted UniFrac) yields a different clustering of samples. Interestingly, one sample from cockatiels remained clustered with budgerigars using both weighted and unweighted UniFrac metrics, suggesting that both metrics do not always imply a different clustering. This study adds valuable information about using the UniFrac phylogenetic method to understand clustering of microbial communities.

Determining the metabolic profile of bacterial communities is hampered by the high numbers of microorganisms and also by the genomic complexity associated with microbial communities. Fortunately, different tools have been developed to predict this profile with some accuracy but very few studies have used them to predict the metabolic profile of bird-associated microbiomes. Waite and Taylor [65] used PICRUSt to predict the metabolic profile associated with the microbiota of many different bird species. Interestingly, these authors focused on several features of interest to cluster types of birds and our results proved to be useful to compare with (Table 3). For example, all of our samples were associated with higher proportions of predicted genes related to carbohydrate metabolism, especially canaries. Waite and Taylor [65] used publicly available sequence data from many different studies while this current study analyzed samples that were collected from the same source (i.e., feces), from caged birds with relatively fixed diets, sequenced and processed in the exact same fashion, thus adding valuable information to the taxonomic composition, and consequently, the metabolic potential of fecal microorganisms from birds. Also, our study reports NSTI scores which are crucial to assess the quality of PICRUSt predictions, something that was not reported in the previous study [65]. Nonetheless, caution must be exerted when interpreting PICRUSt predictions because the functional capacities of multiple bacterial genomes in nature depend on multiple factors (e.g., whole community membership and quorum sensing) and also because PICRUSt can currently only take into account those 16S rRNA gene sequences from very well-defined sequence databases (in this case, the GreenGenes 16S database).

Budgerigars show several quantifiable characteristics that can be used in future studies to test specific hypothesis about their associated microbiota in a context of microbial ecology. Exceptional examples of these sorts of experiments include the relationship between mating and recognition with the gut avian microbiota [73] and the role that bacteria could play in the production and evolution of plumage traits (reviewed in [74]). Dietary changes are also easy to perform using budgerigars [75]. Additionally, budgerigars are cheap to feed and they easily breed, both additional advantages of using budgerigars to test hypotheses. In this study, we show that 12 pooled fecal samples from different pet budgerigars are similar to each other and are mainly dominated by members of Lactobacillaceae and Lachnospiraceae with many other less abundant groups likely occupying important niches in their original environment.

Our research is not exempt of pitfalls and it is important to discuss them to guide future studies. First, this descriptive study used pooled fecal samples, which erases interindividual variability and complicates the statistical analysis. Alternatively, one may separate each bird in a different cage and analyze samples separately, but this approach is associated with higher research costs and more time consuming. Moreover, from a clinical standpoint, there is a possibility of having unnoticeable changes in the bird's physiology compared to the normal physiology of the bird within a flock. Second, this study did not equally sample birds from all three species (this study collected more samples from budgerigars compared to the other species). This was due to the unequal number of pet birds in our area, a phenomenon that has to do with individual preferences as well as social and economic reasons. For example, the colorful budgerigars are the cheapest to buy and feed and therefore are the most common among

Table 3 Summary of key functional features predicted by PICRUSt from our study and the study published by Waite and Taylor [53]

Functional feature	Birds	Proportion of metagenome (%)
Carbohydrate metabolism		
	Wild	10.49
	Herbivore	10.68
	Carnivore	10.85
	Captive	11.28
	Grain-fed	11.51
	Cockatiels	**11.8(11.2–12.8)**
	Budgerigars	**12.7(12–14.1)**
	Canaries	**14.9(10.8–16.2)**
Infectious disease		
	Captive	0.43
	Wild	0.50
	Canaries	**0.52(0.52–0.61)**
	Budgerigars	**0.57(0.55–0.65)**
	Cockatiels	**0.61(0.51–0.65)**
Amino acid metabolism		
	Budgerigars	**7.9(6.8–9.1)**
	Herbivore	8.52
	Grain-fed	8.81
	Canaries	**9.3(8.4–11.8)**
	Cockatiels	**9.9(8.9–10.6)**
	Carnivore	10.86
Signaling molecules and interaction		
	Fecal	0.16
	Crop	0.20
	Illeum	0.23
	Cecum	0.25
	Fecal (Cockatiels)	**0.25(0.2–0.27)**
	Fecal (Canaries)	**0.28(0.19–0.29)**
	Fecal (Budgerigars)	**0.31(0.27–0.35)**

The proportions of metagenome were ordered from lowest to highest and the results of this current study (median (minimum-maximum) are bolded for better visualization

people, while canaries are more expensive than budgerigars but males often sign beautifully, making this specie also popular among people. On the other hand, cockatiels are owned by a much smaller percentage of people in our area due to their larger size, high cost, and typically noisy behavior. Finally, another pitfall of this and other studies on the avian microbiota is that the fecal microbiota from birds does not only come from the gut but some unknown microbial populations are also being shed from the urinary and reproductive tract. This is something very important that has not been discussed even in recent comprehensive reviews on the topic [65, 76, 77]. The fact that feces do not only come from the gut in birds is also important because urine contains a complex microbial consortia in humans [78] and likely in all other animals. Despite these pitfalls, we believe our work provides a foundation for future research on microbial communities associated with pet and other birds.

In summary, this study provides insight into the complex bacterial communities that are present in the feces of birds commonly kept as pets, that these communities cluster separately for each bird species, and that the fecal microbiome possesses a complex metabolic profile that may explain coadaptation to different diets and environments as well as resistance or predisposition to infectious or metabolic diseases. The high abundance of Lactobacillaceae in most samples from canaries and budgerigars may indicate an important role from these organisms perhaps in the distal digestive tract. Importantly, the avian fecal microbiota may not only represent the distal gut microbiota but also other anatomical sites such as the urinary and reproductive tracts. Further studies are needed to evaluate the fecal microbiota and metabolic profile

in more bird species with the hope to aspire for hypothesis-driven experimental work that may help us explain microbial coevolution with avian hosts.

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Compliance with Ethical Standards

Conflicts of Interest The authors declare that they have no conflict of interest.

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