High Efficiency Microbial DNA Extraction Method for Avian Feces and Preen Oil

Austin Russell[1], Alex Van Huynh[2], Margaret Kenna[1], and Amber Rice[1]

[1]Lehigh University
[2]Desales University

October 3, 2023

Abstract

As sequencing technology continues to rapidly improve, studies investigating the microbial communities of host organisms (i.e., microbiomes) are becoming not only more popular but also more financially accessible. Across many taxa, microbiomes can have important impacts on organismal health and fitness. To evaluate the microbial community composition of a particular microbiome, microbial DNA must be successfully extracted. Fecal samples are often easy to collect and are a good source of gut microbial DNA. However, in birds and reptiles, microbial DNA extractions from fecal matter have proven to be difficult due to high concentrations of uric acid, an inhibitor of DNA extractions. Here, we present a new microbial DNA extraction method that is highly effective for avian species and displays higher efficiency and consistency than other commonly used methodologies. Further, our method is also effective in extracting microbial DNA from oils collected from the avian preen gland. Preen oil chemicals are important for many aspects of avian life, and the biosynthesis of these chemicals is dependent on the preen gland microbial community. We expect our method will facilitate microbial DNA extractions from multiple avian microbiome reservoirs, which have previously proved difficult and expensive. Our method therefore increases the feasibility of future studies of avian host microbiomes.

Manuscript Category: Resource Article—*Molecular and Statistical Advances*

Title: **High Efficiency Microbial DNA Extraction Method for Avian Feces and Preen Oil**

Running Title: Avian Microbial DNA Extraction Method

Authors : Austin C. Russell[1,a], Margaret A. Kenna[1], Alex Van Huynh[2], and Amber M. Rice[1]

1. Department of Biological Sciences, Lehigh University, Bethlehem, Pennsylvania, 18015, USA

2. Department of Biology, DeSales University, Center Valley, Pennsylvania, 18034, USA

[a]Corresponding author; email: aar420@lehigh.edu

Abstract

As sequencing technology continues to rapidly improve, studies investigating the microbial communities of host organisms (i.e., microbiomes) are becoming not only more popular but also more financially accessible. Across many taxa, microbiomes can have important impacts on organismal health and fitness. To evaluate the microbial community composition of a particular microbiome, microbial DNA must be successfully extracted. Fecal samples are often easy to collect and are a good source of gut microbial DNA. However, in birds and reptiles, microbial DNA extractions from fecal matter have proven to be difficult due to high concentrations of uric acid, an inhibitor of DNA extractions. Here, we present a new microbial DNA extraction method that is highly effective for avian species and displays higher efficiency and consistency than other commonly used

methodologies. Further, our method is also effective in extracting microbial DNA from oils collected from the avian preen gland. Preen oil chemicals are important for many aspects of avian life, and the biosynthesis of these chemicals is dependent on the preen gland microbial community. We expect our method will facilitate microbial DNA extractions from multiple avian microbiome reservoirs, which have previously proved difficult and expensive. Our method therefore increases the feasibility of future studies of avian host microbiomes.

Keywords: microbiome, Aves, uropygial gland, bacterial symbionts, feces

Introduction

Animal microbiomes have received increased attention in recent years. Further, the natural microbial communities that live on a host organism are known to positively impact health, pathogen resistance, digestive capabilities, and aid in other natural bodily functions. Environment, dietary niche, and the health of a host all influence the diversity and abundance of different bacterial species present in a host organism. Avian species are globally widespread and play many important roles in their ecosystems. Understanding the factors that contribute to health and fitness in birds has become especially important in recent decades due to the rapid decline in bird populations worldwide. Thus, an understanding of the different microbiomes present within and across avian species provides valuable insights about avian health, ecology, evolution, and conservation. A necessary component of characterizing such microbiomes is the accurate and effective identification of microorganisms from specific reservoirs.

Although host species possess many reservoirs for microbial species, one of the most important is the gut. Avian gut microbiomes and their relationship to the behavior and fitness of the host have been of great interest over the past two decades. Specifically, the microbiota that exist in the gut of a bird have been shown to directly influence host behaviors, for example through diet choice and digestion adaptations. Although not yet investigated in birds, gut microbiomes in non-avian taxa are linked to cognition, resistance to disease, increased metabolic functions, and digestion . To study the gut microbiome, most scientists extract bacterial DNA from fecal samples. Because fecal waste begins in the stomach and travels through the entire digestive tract, sequence data obtained from fecal microbial DNA are effective in providing an accurate representation of the abundance and diversity of all the microbial communities existing along the digestive tract.

In addition to gut microbiota, preen gland microbiota are important for birds . The avian preen gland (or uropygial gland) is located above the tail feathers on the back and produces oils that help birds clean themselves, protect feather health, and potentially aid in fighting against pathogenic bacteria . Birds will stimulate this gland with their beaks, and then spread the resulting oil throughout their feathers in a behavior called preening. Besides feather health, chemicals from preen oils vary between species and can mediate communication between individuals in many contexts, including species recognition and mate choice. Additionally, preen oil chemistry has shown to vary with seasonality, aggression, and reproductive success. Interestingly, recent studies have shown that preen oil contains bacteria, which play a role in the synthesis of the chemical compounds found in the oil. Thus, multiple microbial communities from different reservoirs play important roles in avian life.

To characterize the microbiomes present within a species, DNA must be extracted from the microbes present in samples collected from different reservoirs. In order to compare results across studies focusing on different reservoirs, the field would benefit from a microbial DNA extraction method that is effective for different sample types. However, within avian hosts, multiple sample types present particular difficulties. Specifically, microbial DNA extractions from both avian fecal material and preen oil present several challenges. Although feces has abundant bacteria, avian and reptilian organisms combine their feces with urine containing minimal bacteria, lowering the overall concentrations of the bacteria present in fecal samples. Further, urine contains PCR inhibitors such as urea, beta-human chorionic gonadotropin, and crystals that can impede bacterial DNA detection. This combination of digestive and uric acid waste in birds inhibits microbial DNA extraction and subsequent PCR amplification, making it difficult to obtain reliable and consistent results. There are many commercially available kits for fecal extractions, but these can differ in effectiveness and multiple

extractions can quickly become cost prohibitive. Additionally, the preen oil microbiome has only recently begun to receive attention in the literature and there are no standardized methodologies for preen gland microbial DNA extraction.

Most studies on microbiomes have used commercial DNA extraction kits that are used on other forms of eDNA such as feces or soil. For avian fecal matter however, commercial DNA extraction kits are often unsuccessful in producing sufficiently concentrated microbial DNA extractions. For example, Eriksson et al. (2017) compared the performance of six different commercial DNA extraction kits using mallard duck (*Anas platyrhynchos*) feces, and obtained low yield of microbial DNA from all kits [64]. Further, due to the low bacterial abundance present in preen oil[42], we expect similar difficulties in microbial DNA extractions.

Thus, there is a need for more effective, efficient, and inexpensive methodologies for extracting microbial DNA from avian feces and preen oil samples. Here, we present an optimized method for effectively and consistently extracting bacterial DNA from both fecal and preen oil samples collected across a wide range of avian species.

Materials and Methods

1. Fecal and Preen Oil Sampling

We obtained fecal samples from a total of 28 individual birds across 15 species (plus hybrids between black-capped chickadees (*Poecile atricapillus*) and Carolina chickadees (*P. carolinensis*)), 14 genera, 12 families, and 7 orders (Table 1). To capture all but one of the Passerines used in this study, (excluding the American crow (see Table 1)), we used mist nets at feeders in Northampton and Lehigh Counties in Pennsylvania, USA (U.S.G.S. Federal Banding Permits 23810 to AMR and 24256 to AVH; Pennsylvania Banding Permits 103 to AMR and 49864 to AVH). Fecal collections require careful handling to ensure the preservation of DNA. We briefly held each bird individually in a cage containing a clean cage liner until they defecated, which usually took no longer than several minutes. We then immediately collected the feces using tweezers. Tweezers were cleaned with ethanol before and after each use. We transferred the feces into a 1.5 mL microcentrifuge tube containing 100% ethanol and stored the samples in a -80 °C freezer until microbial DNA extraction. Although ethanol kills the living bacteria, it preserves all the DNA present in the sample so that any bacteria present can be detected even after freezer storage[68]. Additionally, in cooperation with the Wildlands Conservancy Nature Preserve in Lehigh County, Pennsylvania, we obtained fecal samples from captive birds including the American crow (*Corvus brachyrhynchos*), plus several species spanning four additional orders (Table 1): red-tailed hawk (*Buteo jamaicensis*), eastern screech owl (*Megascops asio*), turkey vulture (*Cathartes aura*), and rock pigeon (*Columba livia*). The Wildlands Conservancy samples were collected as the individuals were seen defecating in their enclosures. The collections were placed in fresh Ziplock bags rather than in ethanol filled tubes and placed in a freezer prior to extraction. Fecal samples were collected from two budgerigars (*Melopsittacus undulatus*) housed in a local pet store (PetSmart in Bethlehem, Pennsylvania, USA) using sterile tweezers and placed in microcentrifuge tubes containing ethanol as described above. To collect from the Canada goose (*Branta canadensis*), we closely observed wild geese on the DeSales University campus. When we saw defecation, we used sterile tweezers to lift the fresh fecal samples off the ground and placed them into individual tubes of ethanol.

We collected preen oil from a total of 12 passerine species, spanning 10 genera, and 8 families (Table 2). All these birds were captured in mist nets at bird feeders in Lehigh County, Pennsylvania, USA (U.S.G.S Federal Banding Permit 24256 and PA Game Commission Banding Permit 49864). We used 100% ethanol to clean the uropygial gland of each bird, and to temporarily clear away any nearby feathers. We then used small, sterile forceps to gently squeeze the gland. Once preen oil was secreted from the uropygial gland, we used a capillary tube to collect a small oil sample (˜1uL). After collection, the capillary tube was placed in a 1.5 mL microcentrifuge tube and stored in a -80 °C freezer prior to microbial DNA extraction.

All capture and sampling procedures were approved either by Lehigh University's Institutional Animal Care and Use Committee (Protocol #237) or by DeSales University's Institutional Animal Care and Use Committee (Protocol #1).

2. DNA Extractions

We removed approximately 0.15 g feces from each sample, taking care to avoid collecting any of the white uric acid. After pouring any ethanol off the fecal sample, we placed the sample in a weigh boat in a fume hood for about one minute to allow the evaporation of excess ethanol. We then transferred the dried fecal sample into a 2 mL Fisherbrand Free-Standing Microcentrifuge Tube with Screw Cap that had been filled with approximately 100 µL of 460 nm acid-washed glass beads (Sigma-Aldrich, Inc., St. Louis, Missouri, USA) and 200 µL of PrepMan Ultra Sample Preparation Reagent (Thermo Fisher Scientific, Waltham, Massachusetts, USA). For preen oil samples, we placed the entire capillary tube tip (containing the preen oil sample) into a 2 mL microcentrifuge tube containing the same volumes of acid-washed glass beads and PrepMan Ultra Sample Preparation Reagent as used with the fecal samples. Both types of sample mixtures were homogenized using a Mini-Beadbeater (BioSpec Products, Bartlesville, Oklahoma, USA) for 1 minute on high, and then placed in a water bath for 10 minutes at 100°C. For preen oil samples, we removed the capillary tube tip after this water bath step, at which point the oil sample was no longer observable in the tip of the capillary tube. For both sample types, we then centrifuged samples at 14,000 rpm and pipetted off the supernatant. This supernatant was then used as the DNA extract for PCR. The volume of the final DNA extract samples was ˜75µL, depending on how much could be pipetted off without collecting the acid-washed beads. To create a negative control, we followed this protocol using 100 uL of water instead of a dried fecal sample.

3. **PCR Amplification of microbial 16S rRNA**

To assess the success of our microbial DNA extraction method, we performed PCR amplification of hypervariable regions of the 16S rRNA gene (V3-V4 regions) using our DNA extracts from both fecal and preen oil samples. The 16S rRNA gene is commonly used to identify bacterial taxa and quantify microbial diversity. The function of this gene itself has not changed over time, which indicates that the random changes that do exist can be a good measure of evolution and variation. This gene is present in nearly all bacteria and its highly conserved nature coupled with species-specific regions of variation allows for identification of different clades of bacteria.

For most of our DNA extractions from fecal samples, we first diluted 1 µL of the concentrated fecal DNA sample in 99 µL of nuclease free water. If the fecal sample used for microbial extraction was less than 0.1 g, we used a non-diluted DNA extract sample. Since all collected preen samples were ˜1uL, no dilution of the preen oil DNA extracts was needed.

We used a total PCR volume of 20 uL, containing master mix, GC enhancer, forward and reverse primers, DNA, and water. Specifically, each reaction included 10 uL Platinum II Hot-Start Green PCR Master Mix (2X) from Invitrogen (Waltham, Massachusetts), 4uL of the Platinum GC Enhancer included with this master mix, 2uL of nuclease free H_20, and 2uL of a diluted DNA sample. To amplify the V3-V4 region of the 16S rRNA gene, we also included 1uL of 25uM 341F (CCTACGGGNGGCWGCAG) and 806R (GACTCHVGGGTATCTAATCC) 16S rRNA primers.

Optimized PCR conditions included an initial denaturation step at 95°C for 2 min, followed by 30 cycles of denaturation at 95°C for 30 seconds, annealing at 55°C for 30 seconds, extension at 68°C for 1 min, and a final elongation at 68°C for 2 min. To confirm successful PCR amplification, we ran each sample on a 2% agarose gel at 140 V for 50 minutes and verified the presence of bands visually. The expected product size was ˜430 bp.

4. Sequencing Library Preparation

To confirm the amplification of microbial rRNA gene regions from our DNA extracts, we sequenced PCR amplification products from seven individuals of two chickadee species (*Poecile atricapillus* and *P. carolinensis*) and their hybrids. Once we established successful amplification of the V3-V4 regions of the 16S rRNA gene using an agarose gel (see above), we sent seven PCR products and the 16S rRNA primers mentioned above to Rush Genomics and Microbiome Core Facility (Chicago, Illinois, USA) for sequencing.

Library preparation and sequencing of the seven samples were completed by the sequencing facility using the CS1 (*ACACTGACGACATGGTTC-TACA* **CCTACGGGNGGCWGCAG)** and CS2 (*TACGGTAGCA-GAGACTTGGTCT* **GACT-CHVGGGTATCTAATCC)** linkers, indicated by underlining, on the 341F and 806R 16S primers, indicated in bold. The sequencing facility performed Fluidigm amplicon library preparation to ready the samples for next generation sequencing. The samples were normalized, pooled, and sequenced on Illumina MiniSeq at Rush Genomics and Microbiome Core Facility. Quality checks were run on the MiniSeq samples using FastQC. Our seven microbial DNA extracts were sequenced with an Illumina MiSeq using paired end 300 bp reads. With each run, a negative and positive sample were run alongside the samples to control for contaminants at different stages of the sequencing.

5. Sequencing Data Analysis

We adapted a previously published pipeline from the R package 'phyloseq' for the statistical analysis of our microbial sequence data. Once the sequences were obtained from the sequencing facility, we used the program FastQC to check the initial quality of the sequenced samples and to trim primer sequences from the samples. The quality scores indicated the amount of overlap to use for the merged samples. To keep a minimum phred score of 25, we merged the forward sample at 275 bp and cut the reverse at 225 bp. We used the SILVA v138 reference taxonomy dataset to identify the microbial species present within each sample's microbial community. Our R script is available on GitHub (https://github.com/rusty-russ/Russell-et-al.-Methods-Paper).

Results

Our microbial DNA extraction method was successful from both fecal and preen oil samples collected across multiple avian taxa (e.g., songbirds, waterfowl, scavengers, and birds of prey), covering a broad range of feeding guilds (Tables 1 and 2; Figures 1, 2 and 3). The success of our extraction method was evident by our consistent ability to PCR amplify the V3-V4 region of the microbial 16S rRNA gene, indicated by the presence of bands on agarose gels representing PCR products of the expected length (Figures 2 and 3; Supplemental Figures 1-4). Out of the fecal collections from 15 species and preen collections from 11 species, only the preen sample from the song sparrow did not amplify or show on a gel (Tables 3 and 4).

Our successful amplification of the targeted 16S rRNA microbial gene regions was further confirmed by sequencing a subset of our PCR products. Specifically, we sequenced 16S rRNA V3-V4 regions in microbial DNA extractions done with fecal material from black-capped and Carolina chickadees (n=7). We obtained consistently high-quality reads with a mean of 108,000 reads per sample. After trimming and merging the reads, five of seven samples had at least 90% reads retained, while the remaining two had approximately 85% reads retained (see supplemental). As mentioned above, FastQC was used to determine the quality of the reads and maintained a minimum phred score of 25 on both the forward and reverse reads. This resulted in trimming the forward read at 275 bp and the reverse read at 225 bp, allowing some overlap in the merge and higher accuracy on the reads for each sequenced sample. Subsequent bioinformatic analysis of the sequencing results revealed that our extraction method was successful in extracting both gram-positive and gram-negative bacteria (Table 5). Further, our results identifying the bacterial classes present in each extraction, as well as the relative abundance of different bacterial classes, suggests that the gut microbiome can vary across different chickadee individuals (Figure 3).

Discussion

Our new microbial DNA extraction method is effective for avian fecal samples (Figs. 1 and 3; Table 3; Supp. Figs. 1-4) which have been traditionally challenging materials for use in DNA extractions and preen gland oil samples (Fig. 2; Table 4; Supp. Figs. 3-4), both collected across a broad range of avian species. Further, our method successfully extracted DNA from both gram-positive and gram-negative bacteria (Table 5). Additionally, the ease and relative cost effectiveness of our method (Supplemental Table 1) makes it particularly advantageous. We hope that our method will facilitate advances in our understanding of microbiomes from multiple reservoirs across a wide range of avian species.

While the host microbiome of an organism has shown to be a crucial determinant of the overall health of an organism, the full extent of the coevolutionary relationships between a host and its symbiotic microbes is not known. Recent discoveries have shown hosts rely on microbes for health and protection against viruses and pathogens, breaking down compounds, digestion, cognitive function, growth, development, and more. Gut microbiomes are particularly important in many taxa. To sample the gut microbiome of birds, cloacal swabs have been shown to be nonrepresentative of microbial diversity, only accounting for one component of the digestive tract, whereas fecal samples travel the whole digestive tract and are thus accurate representations of the entire gut microbiome. However, existing DNA extraction methods have not been consistently successful when working with avian fecal samples . Birds and reptiles combine their uric waste with their fecal waste, thus creating added difficulty in extracting DNA from fecal samples. Our method is not only reliable with such samples but is considerably cheaper than most commercial kits used to extract avian microbiome DNA (Supplemental Table 1). The low cost and high reliability of our method will improve the feasibility and accessibility of research on avian host microbiomes.

As microbiome studies have increased popularity in the last decade, the host reservoirs in which scientists have been studying has expanded as well. However, beyond the gut, the community composition of microbiomes from additional reservoirs in host animals needs to be explored before the function of these bacterial communities can be investigated as well. Beyond the abundant and diverse gut microbiome, a potentially functionally important reservoir in avian species is the microbiome that exists within the uropygial or preen gland. The preen gland and the oils produced there are known to affect communication, species recognition, mate choice, and feather maintenance. The chemical composition of preen oil is at least partially dependent on the microbes present in this gland. The bacterial communities present in the uropygial gland have been shown to be less diverse and abundant than those within the gut microbiome; yet, they are predicted to play direct roles in the odor of avian hosts as well as potentially in feather and body health. Some preliminary studies in this field of research have observed that disease may not be directly linked to differences in preen oil microbiomes. Additionally, differences in microbial communities may correspond with population differences, and in some species, sex differences have been observed. The consistent success of our method in extracting microbial DNA from avian preen oil samples will facilitate new research on this currently understudied microbiome reservoir.

Because we have established the consistent success of our method in extracting microbial DNA from two avian sample types that are quite different, it is likely that our method will be useful across additional avian microbiome reservoirs. Using the same extraction methodology will provide more consistent and reliable results for future research efforts. Standardization of methodology is important to allow for accurate cross-study comparisons and higher confidence in the results of individual studies. Current variation in the approaches used for microbial DNA extraction and subsequent amplification means there is also sometimes variation in the bacteria that are detected. Widespread adoption of our method would increase the validity of future cross-study comparisons.

With increased interest in the relationship between hosts and their bacterial community composition and abundance across different microbiome reservoirs, the accurate identification of microbial taxa is critical. With an enhanced potential to identify and quantify the symbiotic microbes, the investigation of their impact on host health and conservation can involve deeper questions. To conclude, we have developed and tested a highly efficient microbial extraction method and verified its efficacy across multiple avian species. Our method was successful in extracting microbial DNA from the fecal samples of 15 avian species (Table 1, Figure 1, Supplemental Figures 1-4) and the preen oil samples of 11 avian species (Table 2, Figure 2, Supplemental Figures 3-4). From this, we are confident that our method will be effective and efficient across many additional avian species globally. Future work should explore the effectiveness of this method in extracting microbial DNA from reptile fecal samples as well.

Acknowledgements

This research was supported by the U.S. National Science Foundation (NSF IOS-1754909 and DEB-1928802 to A.M.R.) and Lehigh University. We thank Luke McCartin, Nicole Pittoors and Samuel Vohsen for their

assistance with microbial sequencing and bioinformatics; and we thank Joan Spinelli, Noel Martinez, Ray Kennedy, and Imani Ashman for providing feedback on an earlier draft of this paper. We also thank Brandon Swayser and Haley D'Agostino at the Wildlands Conservancy for collecting fecal samples.

References

Data Accessibility Statement

Genetic data:

Raw sequence reads are deposited in the NCBI Nucleotide Database (PRJNA1015692)

Sample metadata:

Metadata are also stored in NCBI Nucleotide Database (PRJNA1015692)

Benefit-Sharing Statement

Benefits Generated: Benefits from this research accrue from the sharing of our data and results on public databases as described above.

Author Contributions

ACR designed the experiment, optimized the method, collected, and analyzed data, and wrote and edited the manuscript. MAK optimized the method and edited the manuscript. AVH collected data and edited the manuscript. AMR designed the experiment, edited the manuscript and secured the funding.

Tables and Figures

Table 1 : Avian taxa from which we obtained fecal samples.

Class	Order	Family	Genus	Species	Common Name	Feeding Gu
Aves	Anseriformes	Anatidae	Branta	*Branta canadensis*	Canada goose	Granivore, G
Aves	Columbiformes	Columbidae	Columba	*Columba livia*	Rock pigeon	Granivore
Aves	Accipitriformes	Cathartidae	Cathartes	*Cathartes aura*	Turkey vulture	Scavenger
Aves	Accipitriformes	Accipitridae	Buteo	*Buteo jamaicensis*	Red-tailed hawk	Carnivore
Aves	Piciformes	Picidae	Dryobates	*Picoides pubescens*	Downy woodpecker	Insectivore
Aves	Strigiformes	Strigidae	Megascops	*Megascops asio*	Eastern screech owl	Carnivore
Aves	Psittaciformes	Psittaculidae	Psittacula	*Melopsittacus undulatus*	Budgerigar	Frugivore, G
Aves	Passeriformes	Corvidae	Corvus	*Corvus brachyrhynchos*	American crow	Omnivore
Aves	Passeriformes	Paridae	Baeolophus	*Baeolophus bicolor*	Tufted titmouse	Insectivore
Aves	Passeriformes	Paridae	Poecile	*Poecile carolinensis*	Carolina chickadee	Insectivore, I
Aves	Passeriformes	Paridae	Poecile	*Poecile atricapillus*	Black-capped chickadee	Insectivore, I
Aves	Passeriformes	Sittidae	Sitta	*Sitta carolinensis*	White breasted nuthatch	Insectivore
Aves	Passeriformes	Icteridae	Molothrus	*Molothrus ater*	Brown-headed cowbird	Granivore
Aves	Passeriformes	Passerellidae	Junco	*Junco hyemalis*	Dark-eyed junco	Granivore
Aves	Passeriformes	Passerellidae	Melospiza	*Melospiza melodia*	Song sparrow	Insectivore

Table 2 : Avian taxa from which we obtained preen oil samples.

Order	Family	Genus	Species	Common Name
Passeriformes	Corvidae	Corvus	*Corvus brachyrhynchos*	American crow
Passeriformes	Paridae	Poecile	*Poecile carolinensis*	Carolina chickadee
Passeriformes	Paridae	Poecile	*Poecile atricapillus*	Black-capped chickadee

Order	**Family**	**Genus**	**Species**	**Common Name**
Passeriformes	Turdidae	Turdus	*Turdus migratorius*	American robin
Passeriformes	Sittidae	Sitta	*Sitta carolinensis*	White-breasted nuthatch
Passeriformes	Fringillidae	Carduelis	*Carduelis tristis*	American goldfinch
Passeriformes	Fringillidae	Carpodacus	*Carpodacus mexicanus*	House finch
Passeriformes	Cardinalidae	Cardinalis	*Cardinalis cardinalis*	Northern cardinal
Passeriformes	Passerellidae	Zonotrichia	*Zonotrichia albicollis*	White-throated sparrow
Passeriformes	Passerellidae	Melospiza	*Melospiza melodia*	Song sparrow
Passeriformes	Icteridae	Agelaius	*Agelaius phoeniceus*	Red-winged blackbird
Passeriformes	Icteridae	Molothrus	*Molothrus ater*	Brown-headed cowbird

Table 3 : Summary of gel evidence showing successful PCR amplification of the V3-V4 region of the 16S rRNA gene using microbial DNA extracted from the fecal samples of the indicated species. Cells filled with black indicate amplified gene products from the species were not run on that gel.

Species	*Common Name*	*Fig1*	*SupFig1*	*SupFig2*	*SupFig3*	*SupFig4*
Branta canadensis	Canada Goose	X		X	X	
Columba livia	Rock Pigeon		X	X		
Cathartes aura	Turkey vulture	X		X		X
Buteo jamaicensis	Red-tailed hawk	X		X		X
Picoides pubescens	Downy Woodpecker	X		X	X	
Megascops asio	Eastern screech owl			X		
Melospittacus undulatus	Budgerigar	X		X		X
Corvus brachyrhynchos	American crow	X		X		
Baeolophus bicolor	Tufted titmouse		X		X	
Poecile carolinensis	Carolina chickadee	X		X	X	
Poecile atricapilus	Black-capped chickadee	X			X	
Sitta carolinensis	White breasted nuthatch		X	X	X	
Molothrus ater	Brown-headed cowbird		X			
Junco hyemalis	Dark-eyed junco	X		X	X	
Melospiza melodia	Song sparrow	X		X		

Table 4 : Summary of gel evidence showing successful PCR amplification of the V3-V4 region of the 16S rRNA gene using microbial DNA extractions from the preen oil samples of the indicated species. The PCR product from the song sparrow preen oil microbial DNA extract was the only PCR product that did not appear on any gel. Cells filled with black indicate amplified gene products from the species were not run on that gel.

Species	*Common Name*	*Fig2*	*SupFig3*	*SupFig4*
Cyanocitta cristata	Blue jay			X
Poecile carolinensis	Carolina chickadee	X	X	
Poecile atricapillus	Black-capped chickadee	X		
Turdus migratorius	American robin	X	X	
Sitta carolinensis	White-breasted nuthatch	X	X	
Carduelis tristis	American goldfinch	X	X	
Carpodacus mexicanus	House finch	X	X	
Cardinalis cardinalis	Northern cardinal	X		X
Zonotrichia albicollis	White-throated sparrow	X	X	

Species	*Common Name*	*Fig2*	*SupFig3*	*SupFig4*
Melospiza melodia	Song sparrow			
Agelaius phoeniceus	Red-winged blackbird	X	X	
Molothrus ater	Brown-headed cowbird	X		

Table 5 : Gram-positive and gram-negative categorization of the bacterial taxa detected from the amplification of the 16S rRNA gene from seven microbial DNA samples extracted using our method.

Gram-Positive	**Gram-Negative**
Acidimicrobiia	Acidobacteriae
Alphaproteobacteria	Alphaproteobacteria
Actinobacteria	Bacterioidia
Bacilli	Chloroflexia
Clostridia	Cyanobacteriia
Thermoleophilia	Fusobacteriia
Vicinamibacteria	Gammaproteobacteria
	Gemmatimonadetes
	Negativicutes
	Oligoflexia
	Plantomycetes
	Verrucomicrobiae

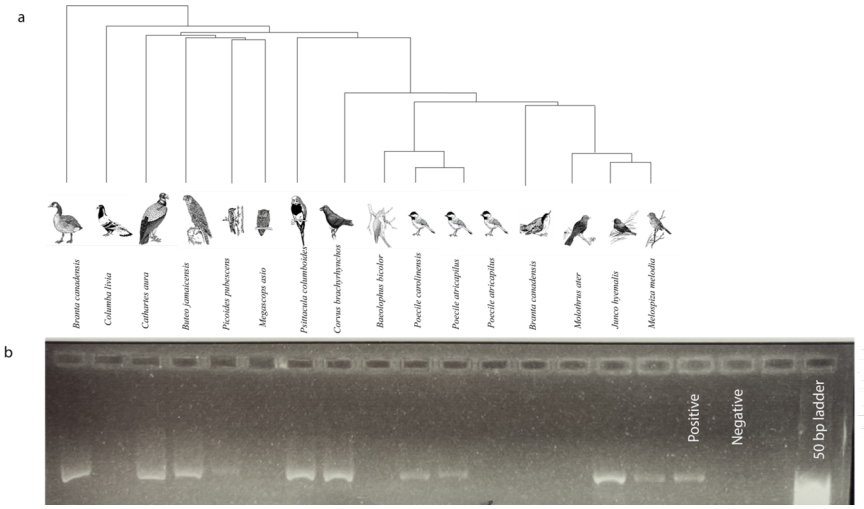

Figure 1 : (a) Phylogenetic relationships of the avian taxa from which we obtained fecal samples. The phylogenetic tree was made using Vertlife.org. (b) Agarose gel showing bands produced by PCR amplification of the V3-V4 regions of the 16S rRNA gene, using microbial DNA extracted from fecal samples following our new protocol. The lanes of the 2% agarose gel pictured in (b) contain the PCR product from the fecal microbiome of the avian species pictured directly above in the phylogeny (a). The negative control, an E coli positive control, as well as a 50bp ladder are also included in the agarose gel. For samples lacking bands in this gel, see supplementary figures.

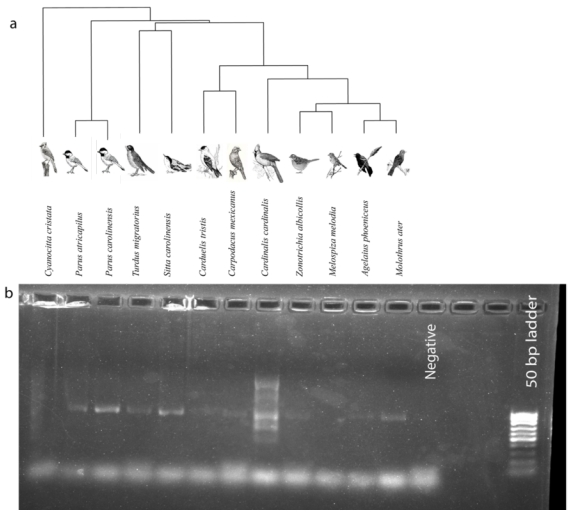

Figure 2 : (a) Phylogenetic relationships of the avian taxa from which we obtained preen oil samples. The phylogenetic tree was made using Vertlife.org. (b) Agarose gel showing bands produced by PCR amplification of the V3-V4 regions of the 16S rRNA gene, using microbial DNA extracted from preen oil samples following our new protocol. The lanes of the 2% agarose gel pictured in (b) contain the PCR product from the uropygial gland microbiome of the avian species pictured directly above in the phylogeny (a). The negative control as well as a 50bp ladder are also included in the agarose gel. For samples lacking bands in this gel, see supplementary figures. Preen oil from the song sparrow was the only sample for which a band from PCR amplification of the V3-V4 regions of the 16S rRNA gene did not appear on any gel.

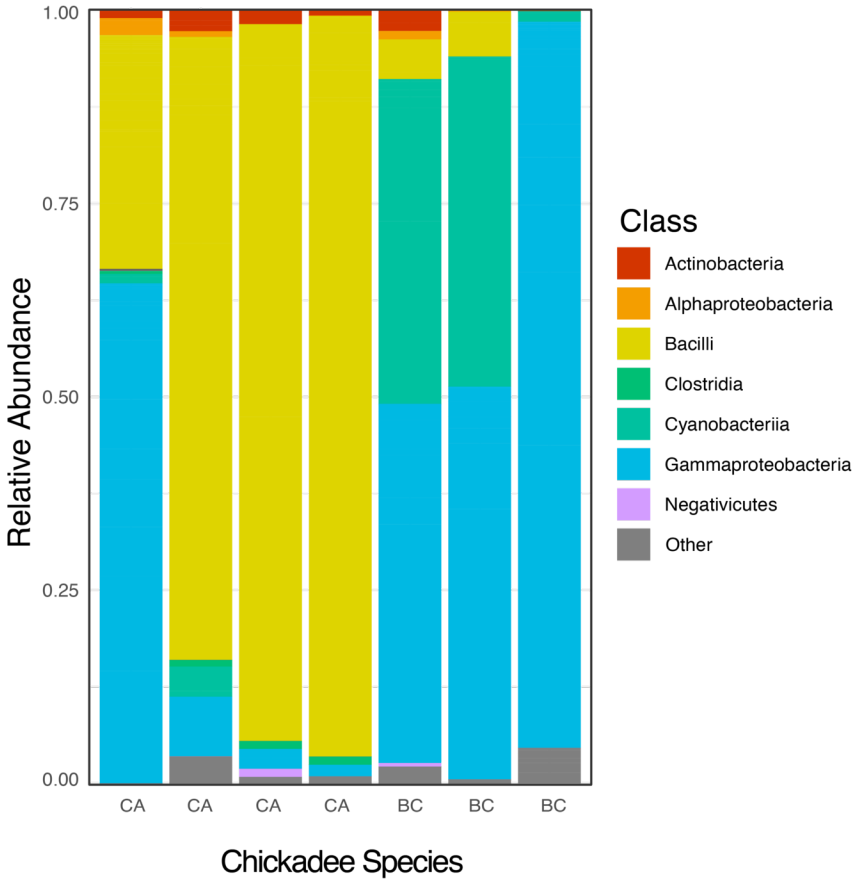

Figure 3 : Relative abundance of several bacterial classes present in the microbial DNA extracted from black-capped (BC;*Poecile atricapillus*) and Carolina chickadee (CA; *P. carolinensis*) fecal samples. See text for an explanation of how the microbial species present were identified using sequence data.