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AN INITIAL DESCRIPTION OF THE FECAL MICROBIOME OF WINTERING NORTHERN SHRIKES *LANIUS BOREALIS*

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Abstract.
This study aimed to broaden our understanding of the natural history of Northern Shrikes by analyzing the gut microbiome of wintering individuals in northern Minnesota using fecal samples from 19 individuals. Bacterial DNA was extracted and sequenced using Illumina metabarcoding subsequently processing these reads via QIIME2. The microbiome was dominated by the phyla Proteobacteria (88.8%) and Firmicutes (9.9%), two phyla often found in high proportions in avian gut samples. However, the preponderance of Proteobacteria is noteworthy but may be explained by the sample from one individual, an adult male. Notable genera included Rickettsiella, Clostridioides, and Lactobacillus. We found no statistically significant differences in microbiome evenness and diversity between sexes or age groups. This study provides one of the first insights into the microbiome of Northern Shrikes, contributing valuable data on this understudied species. Further research is recommended to investigate microbiome variations across Laniidae geographically, with respect to diet, and among different habitats.

Keywords: *Northern Shrike;* Lanius borealis*; winter; microbiome; metabarcoding; QIIME2; fecal microbiome*

Introduction

Between 1970 and 2017, populations of birds breeding in North American Arctic tundra have declined nearly 25% (Rosenberg et al. 2016). The estimated loss of Northern Shrikes *Lanius borealis* amounts to over 96000 individuals of an estimated continental population during this time period of 400000 (100000-700000 estimated range) individuals. Clearly, along with Loggerhead Shrikes *L. ludovicianus*, these predatory songbirds are suffering greatly, a family-wide trend noted many years ago following the convening of the 1st International Shrike Symposium (Yosef and Lohrer 1995). Furthermore, thirty-years following that first symposium little information yet exists on the natural history, demographic parameters, and population trends of Northern Shrikes breeding across northern Canada and Alaska as this species breeds in difficult to access locations and habitats (riparian zones along Arctic and subarctic rivers flowing through tundra and muskeg bogs along the ecotone of the boreal forest and this tundra), has likely never been common, and is labor intensive to capture on the wintering grounds spending its time in open landscapes occupying large winter territories (Atkinson 1993, Atkinson and Cade 1993). Northern Shrike wintering numbers are cyclic with low predictability in site occupancy from year to year (Rimmer. and Darmstadt 1996, Atkinson 1995, ECA unpub. data). That said, substantial efforts have been made to understand the natural history of American Northern Shrikes with often an unspoken desire to determine population status, trends, and limits to this wondrous bird that overlaps in its wintering range with the northern breeding range of its smaller congener the Loggerhead Shrike. These efforts include studies on metabolism (Paruk et al. 2015), morphology and sexual identification (Brady et al. 2009), breeding biology (Cade and Swem 1995), and hunting behavior (Atkinson 1997); see Paruk et al. (2020).

In this study, we wished to broaden the basic natural history information available for Northern Shrikes wintering in the northern United States. Specifically, with birds in-hand, an increased effort in sampling can generate large dividends including the characterization of the gut microbiome. Little information on the microbiome of shrikes exists (see Negruțiu et al. 2017, Hu et al. 2022) and the paucity of such information leaves a substantial gap in our understanding of this family. For that reason, we collected and sequenced the microbiome of all birds, including shrikes, captured, processed, and banded before release. Herein, we describe to our knowledge the first metabarcoding of wild Northern Shrikes in North America.

Materials and Methods

We captured Northern Shrikes in St. Louis, Aitkin, and Carlton Counties of northern Minnesota, USA (47°12'N, 92°37'W) between the months of December-March during the winters of 2021-2022 and 2022-2023.

We located shrikes via extensive driving through suitable habitat while scanning for shrikes hunting near roads.

Shrikes were trapped using a round potter trap (in the style of Craig, 1997) or small bal-chatri with a noose carpet baited with a live mouse. For each shrike, we recorded age, fat score, mass, wing chord, tail length, and extent of black on the outermost rectrix (r6). These morphometric measurements were used to determine sex of adults using the discriminant function equation developed by Brady et al. (2009). All birds were captured as part of a larger study on breeding origins and migratory movements of northern shrikes in the western Great Lakes region.

While in-hand, we aseptically recovered fecal samples immediately placing them in 2 ml cryovials which were then kept cool. Samples were refrigerated (4-8° C) and subsequently frozen at -80°C before extracting DNA. Staff at University of New Hampshire's Hubbard Center for Genome Studies (HCGS) extracted fecal DNA and amplified (PCR) on the v4/v5 gene of the ribosomal RNA with the primers 16S 515 (forward) and 16S 926 (reverse). Primers 515F–806R target the V4 region of the 16S SSU rRNA. Paired ends sequencing of 250 bases was performed on an Illumina NovaSeq SP PE 250. Following the QIIME2 metabarcoding pipeline (Kuczynski et al. 2012. Allali et al. 2017, Bolyen et al. 2019) we trimmed primers from the leading ends and truncated the sequences at positions 247 and 246 for the forward and reverse reads, respectively, based upon the 25th percentiles corresponding to Phred scores (a measure of quality) greater than 25 (Figure 1). To be clear, we followed the protocols outlined by Estaki et al. (2020 https://curr-protoc-bioinformatics.qiime2.org/)

As DNA sequences showed overall good quality post-trimming and truncating, we were satisfied that they represented the bacterial communities contained within Northern Shrike fecal samples well. Hence, we produced box and whisker plots of both bacterial taxonomic evenness (Pielou 1966) and bacterial diversity, testing for differences in age-class and sex in our sampled shrikes via QIIME2view (https://view.qiime2.org/). To be more comparable to ecological studies and to increase interpretability to ecologists of varied backgrounds we calculated Shannon Entropy that assesses disorder (Shannon and Weaver 1949) in addition to the more recently derived Faith's phylogenetic diversity (henceforth, Faith's PD) that accounts for phylogenetic tree branch length when calculating alpha diversity (Faith 1992). Furthermore, in this paper, we acknowledge the use of operational taxonomic units (OTU) rather than using the term 'species' to describe taxonomically identifiable organisms in our samples. We performed Kruskal-Wallace tests, within QIIME2, for differences between identified sex and age of the shrikes across Evenness, Shannon Entropy, and Faith's Phylogenetic PD.

Results

We captured 37 Northern Shrikes and recovered fecal samples yielding extractable DNA from 19 individuals; five females, eight males, six unknown sex; six (HY and early SY) first-year birds and 13 birds greater than one-year of age.

The vast majority (99.8%) of bacterial types identified in Northern Shrike fecal material spanned four bacterial phyla as outlined in the taxa barplots depicted in Figure 2 and Figure 3. Phyla Proteobacteria contributed 88.8% of reads, Firmicutes 9.9%, Actinobacteriota 0.9%, and Desulfobacterota 0.2 (Figure 4). By far, most bacteria enumerated were of phylum Proteobacteria, a very diverse, ubiquitous, and common type of prokaryote.

At the genus-level, *Rickettsiella*, *Clostridioides*, *Sporosarcina*, and *Lactobacillus* predominated in the fecal samples (Figure 5, Table 1). *Rickettsiella*, an outlier, was only found in two shrikes.

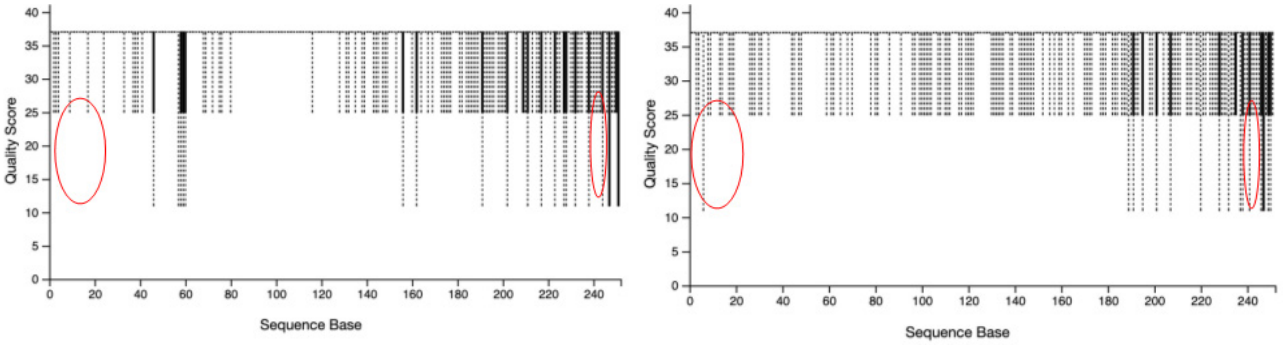

Figure 1. Quality plots of Forward Reads (top) and Reverse Reads (bottom). Areas of trimming (left on each graph) and truncation (right on each graph) are circled.

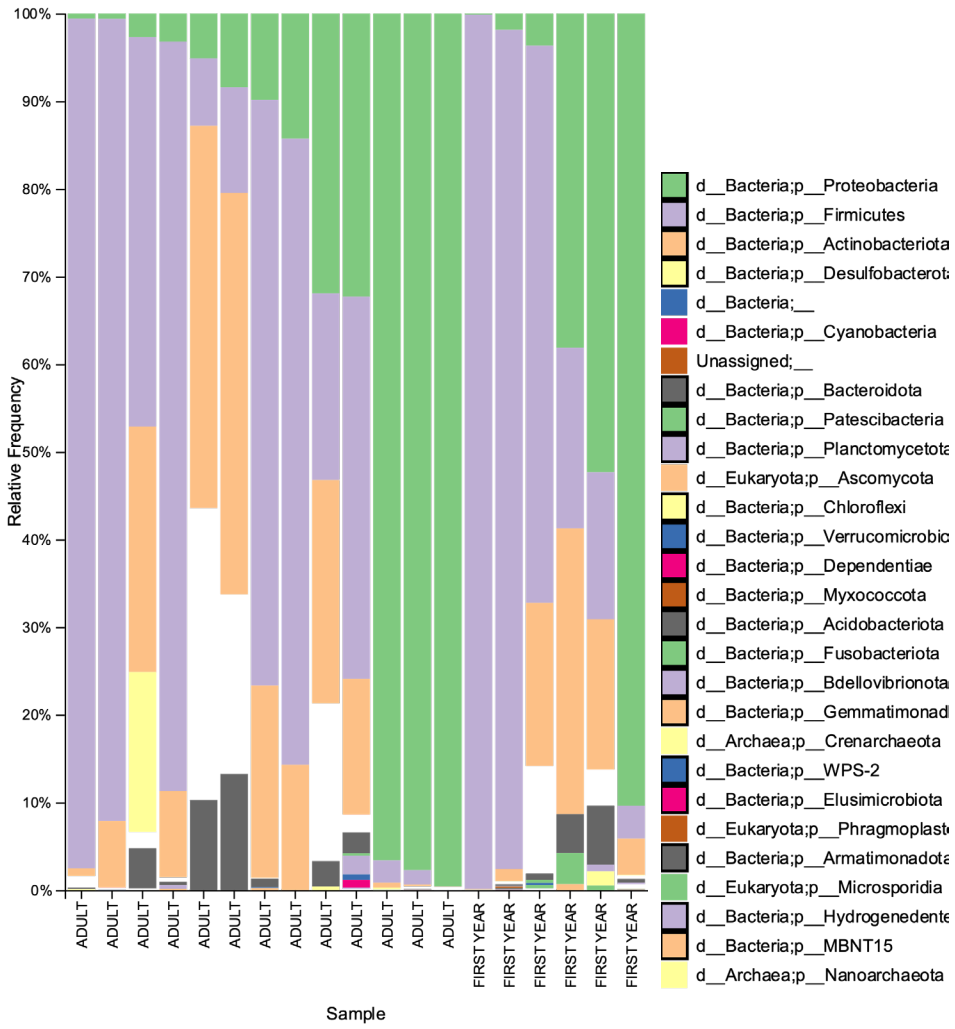

Figure 2. Taxa bar plot, level 2 (phylum) of bacterial (some Eukarya and Archaea) taxa sequenced from Northern Shrike fecal samples.

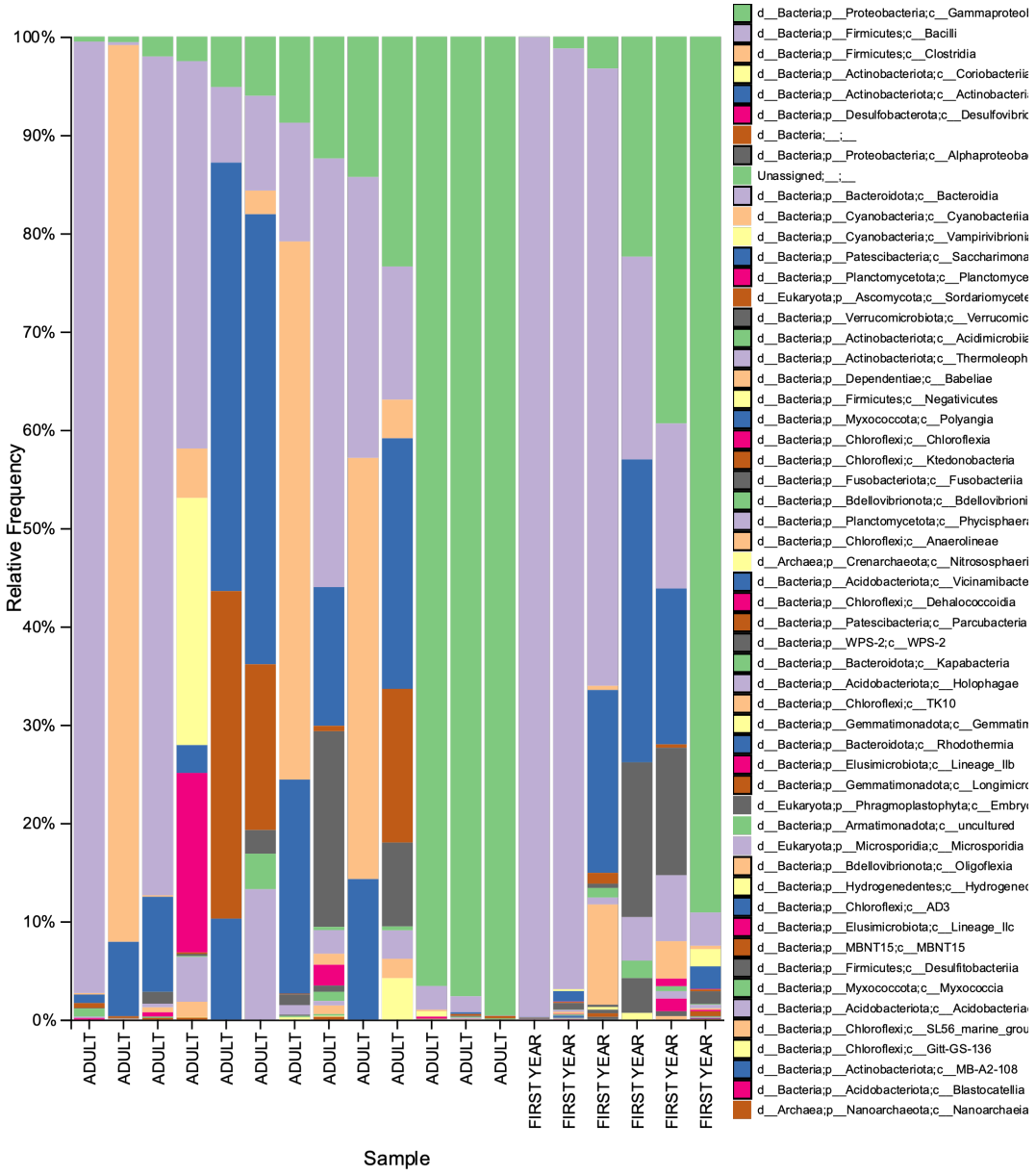

Figure 3. Taxa bar plot, level 3 (class) of bacterial (some Eukarya and Archaea) taxa sequenced from Northern Shrike fecal samples.

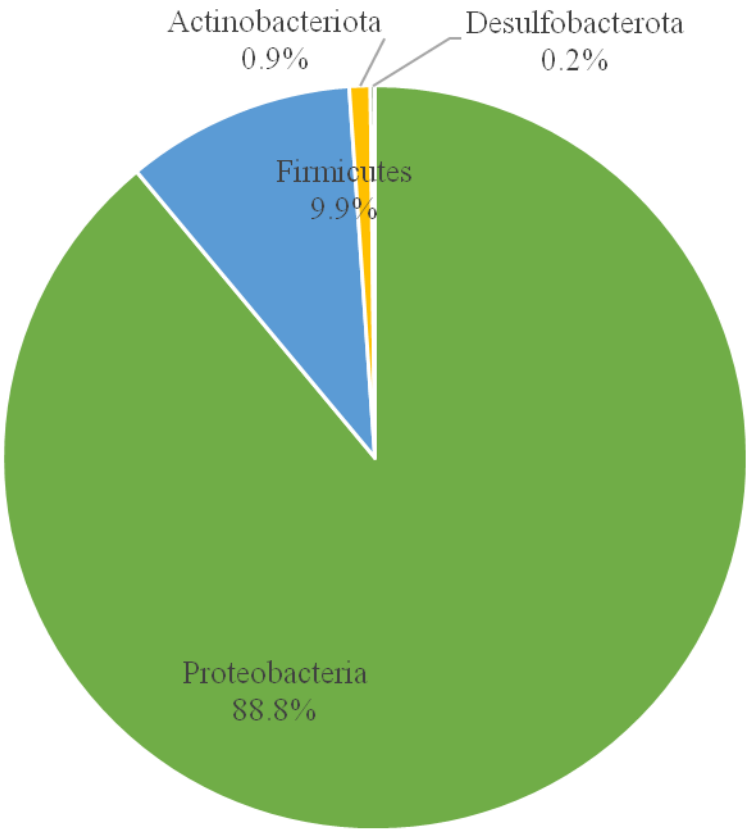

Figure 4. Proportional composition of four most common bacterial phyla sequenced from Northern Shrike fecal samples based upon number of reads.

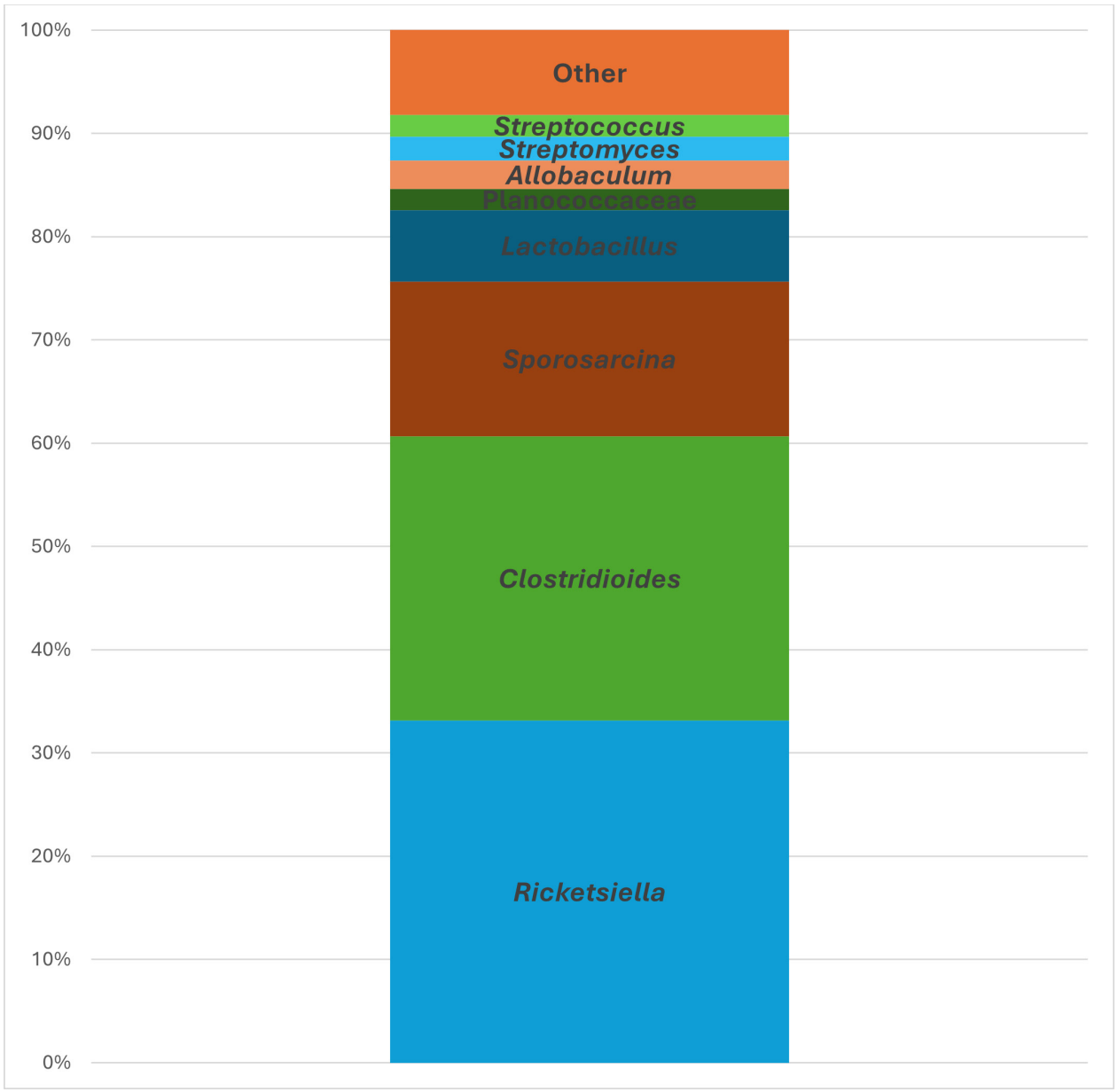

Figure 5. Proportional composition of most abundant bacterial genera sequenced from Northern Shrike fecal samples based upon number of reads.

Table 1. Some bacterial genera and/or species (OTUs) of interest. *n* = 19 Northern Shrikes.

OTU (Species)	Shrike #	Read #	Notes
Pseudomonas sp.	16	6522421	This genus is speciose (> 300 spp) ubiquitous and found in substantial numbers in three shrikes (2 F, 1 U) all captured in 2022
Diplorickettsia sp.	16	437813	Intracellular parasite of Ixodes ticks.
Clostridioides mangenotii	16	364136	Intestinal anaerobic bacterium
Lactobacillus rodentium	18	40800	Intestinal symbiont of rodents
Streptomyces sp.	15	30187	Ubiquitous bacterium
Streptococcus sp.	6	22019	Ubiquitous aerobic intestinal bacterium
Bacillus sp.	8	11983	Ubiquitous bacterium
Desulfovibrio sp.	15	12728	Sulfate reducing Gram-negative bacterium often associated with high organic material (i.e., fens and bogs)
Adlercreutzia equolifaciens	16	2511	Anti-inflamatory commensal intestinal bacterium
Rickettsiella sp.	2	585	Arthropod intracellular parasite, often of Ixodes ticks.
Yersinia sp.	1	189	Yersinia, the causative agent of sylvatic plague (but many species within this genus) was found in the feces of one Female shrike captured in 2022. Species identification was indeterminate (and may not be Y. pestis).
Coxiella sp.	1	2	Coxiella, causative agent of Q Fever both aerosolized and tick-borne. Found in a Male trapped in 2023.
Haemophilus influenzae	1	11	Haemophilus influenzae found in a Female trapped in 2023. This taxon is ubiquitous and may cause a range of nonserious to serious diseases.
Corynebacterium tuberculostearicum	3	156	Lipophilic Gram positive rod inhabiting mucousal membranes.
Legionella sp.	3	132	Potentially pathogenic genus found in aquatic environments

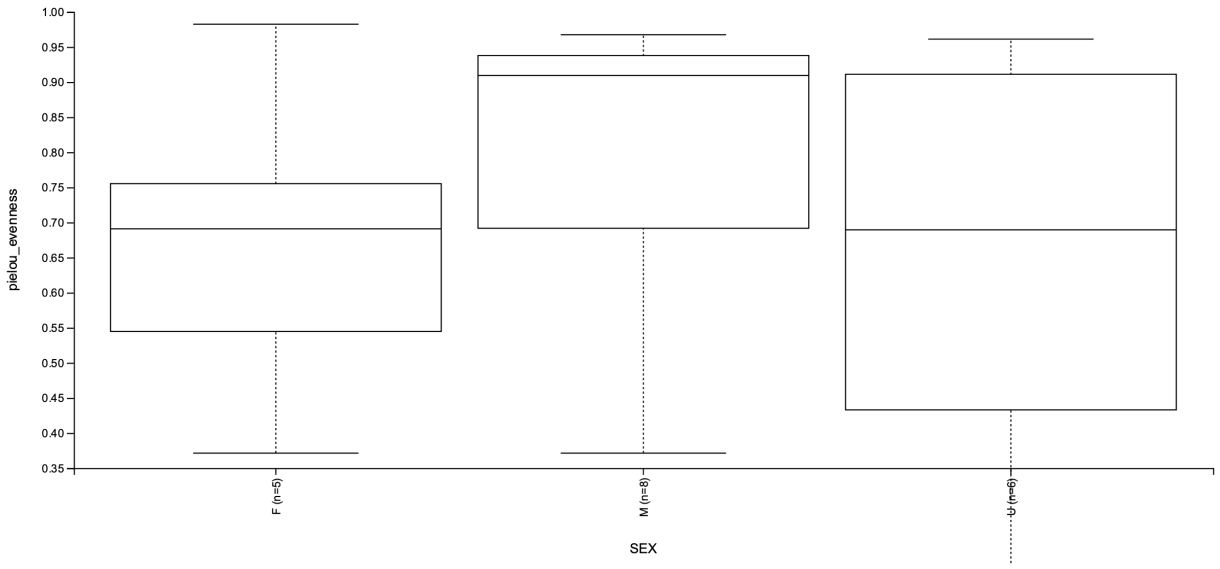

Figure 6. Comparison of Pielou's phylogenetic evenness (by sex of shrike). Evenness does not statistically differ between sexes. Line indicates median with lower and upper quartiles denoted by box, whiskers indicate 1.5 times interquartile range. No significant differences between sex (Kruskal-Wallace test, $H = 1.3579$, df = 2, $P = 0.5072$).

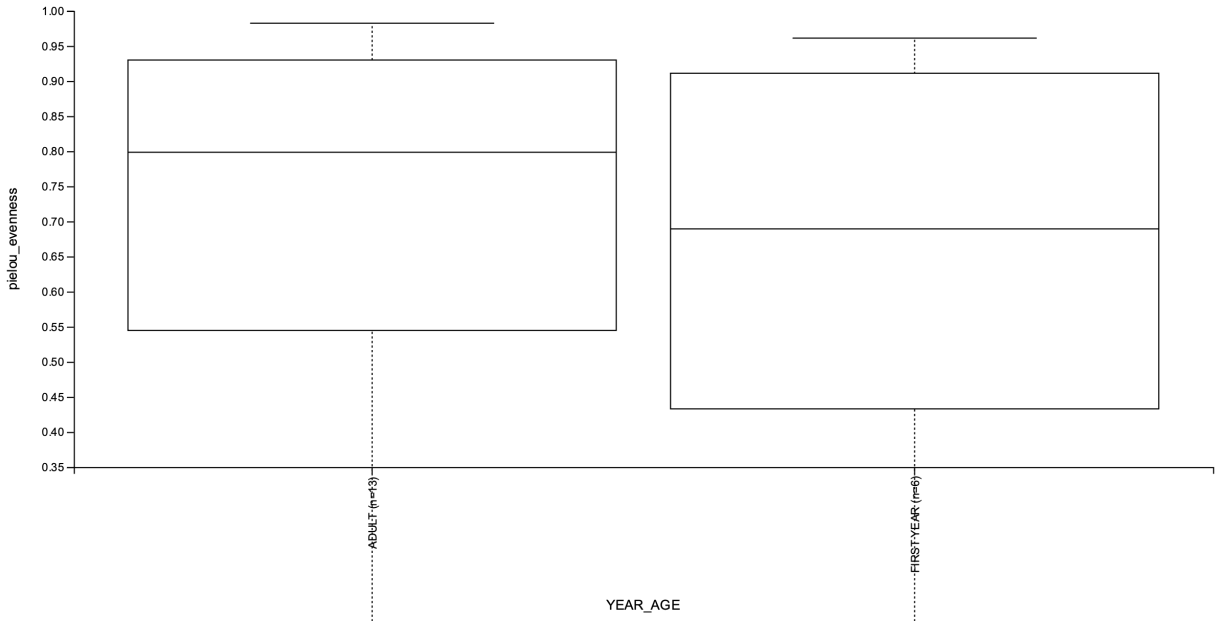

Figure 7. Comparison of Pielou's phylogenetic evenness (by age of shrike). Evenness does not statistically differ between ages. Line indicates median with lower and upper quartiles denoted by box, whiskers indicate 1.5 times interquartile range. No significant differences between age (Kruskal-Wallace test, $H = 0.0449$, df = 1, $P = 0.8322$).

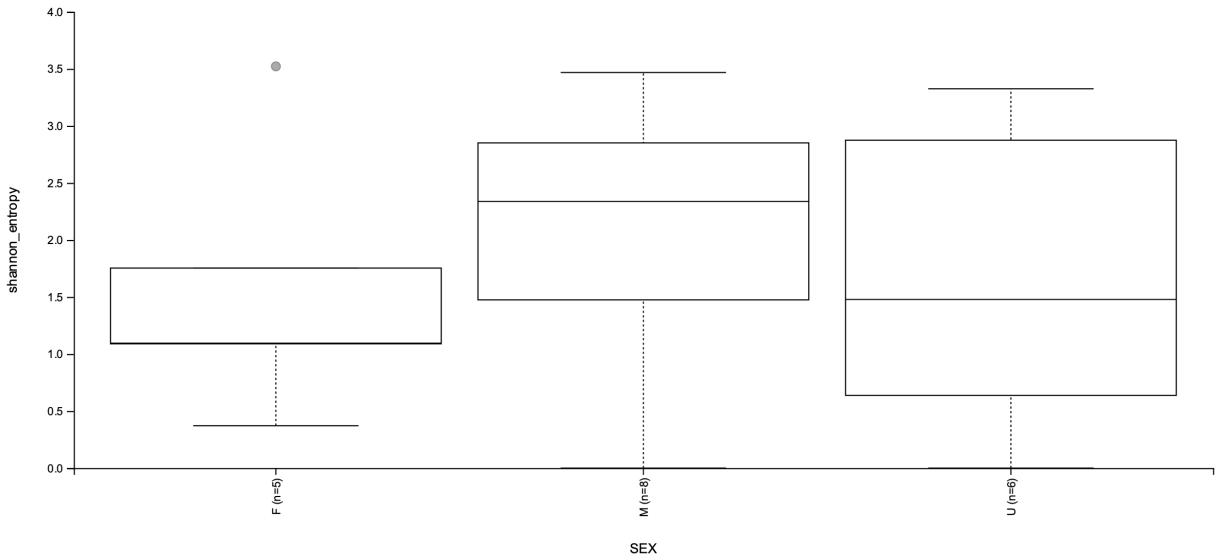

Figure 8. Comparison of alpha phylogenetic diversity (by sex of shrike) as estimated by Shannon Entropy. Alpha diversity does not statistically differ between sexes. Line indicates median with lower and upper quartiles denoted by box, whiskers indicate 1.5 times interquartile range. Dot denotes likely outlier. No significant differences between sex (Kruskal-Wallace test, $H = 0.6214$, df = 2, $P = 0.7329$).

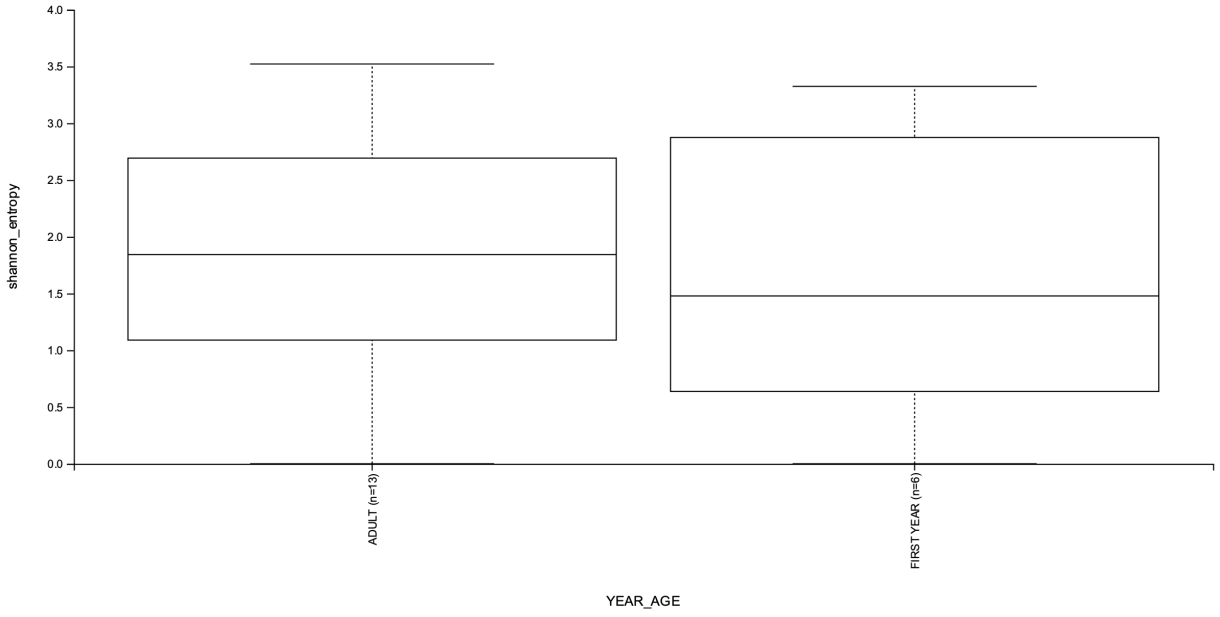

Figure 9. Comparison of alpha phylogenetic diversity (by age of shrike) as estimated by Shannon Entropy. Alpha diversity does not statistically differ between sexes. Line indicates median with lower and upper quartiles denoted by box, whiskers indicate 1.5 times interquartile range. No significant differences between age (Kruskal-Wallace test, $H = 0.2345$, df = 1, $P = 0.6282$).

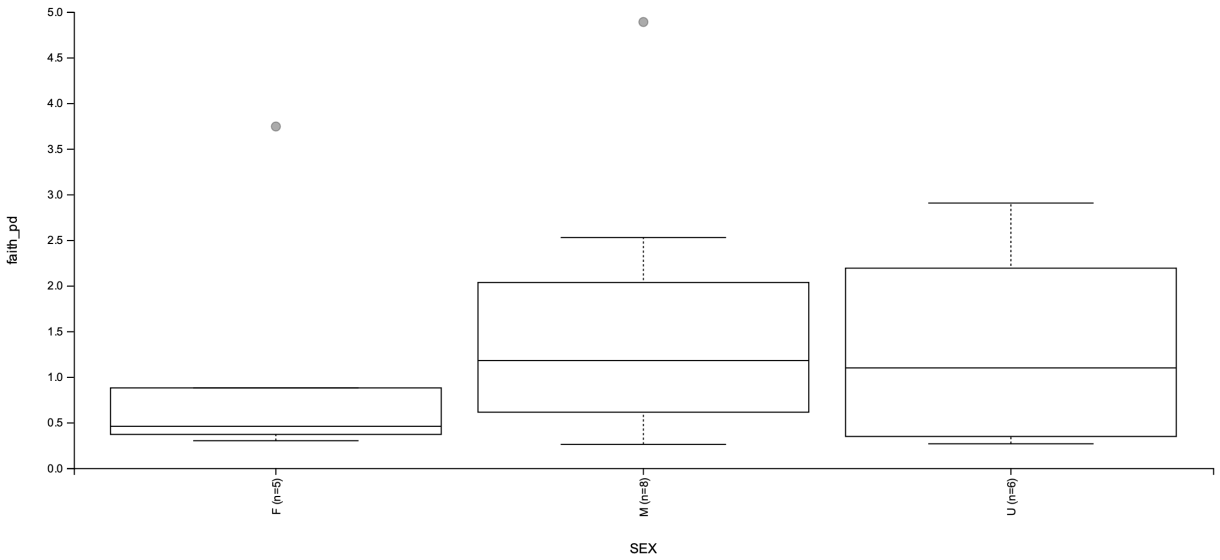

Figure 10. Comparison of alpha phylogenetic diversity (by sex of shrike) as estimated by Faith's PD. Alpha diversity does not statistically differ between sexes. Line indicates median with lower and upper quartiles denoted by box, whiskers indicate 1.5 times interquartile range. Dots denote likely outlier. No significant differences between sex (Kruskal-Wallace test, $H = 0.1766$, df = 2, $P = 0.9155$).

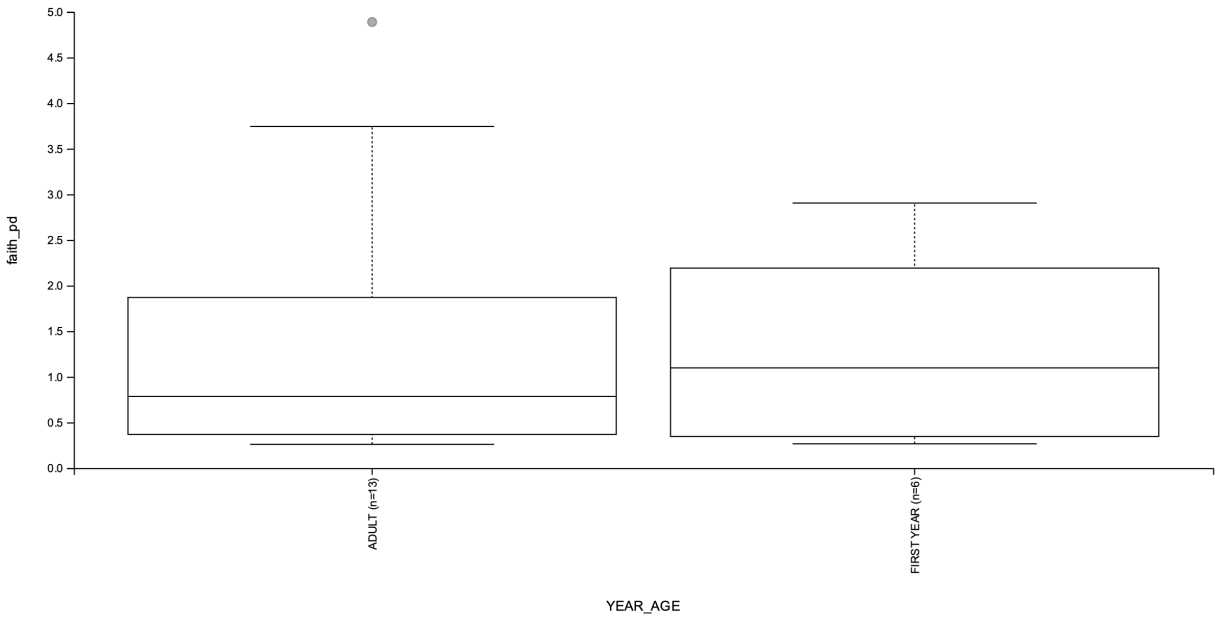

Figure 11. Comparison of alpha phylogenetic diversity (by age of shrike) as estimated by Faith's PD. Alpha diversity does not statistically differ between age. Line indicates median with lower and upper quartiles denoted by box, whiskers indicate 1.5 times interquartile range. Dot denotes likely outlier. No significant differences between age (Kruskal-Wallace test, $H = 0.0308$, df = 1, $P = 0.8608$).

Discussion

Herein, we describe one of the first samplings of Northern Shrike microbiomes. Taken from fecal material, the wintering Northern Shrike microbiome compares well with that of other wild passerines with a heavy preponderance of Proteobacteria, Firmicutes, and Actinobacteriota (88.8%, 9.9%, and 0.9%, respectfully). However, our results notably depart from the common four bacterial phyla in that we identified few bacteria within Phylum Bacteroidetes (0.015%). (Waite and Taylor 2014, Grond et al. 2018). Many taxa were types of bacteria largely associated with soils and aquatic environments likely corresponding with their habitat and dietary associations (Atkinson and Cade 1993). In a pattern similar to that described by Hird et al. (2015) Northern Shrike samples appear to be enriched in Proteobacteria with low Actinobacteriota and Bacteroidetes. We believe that one shrike in particular may have skewed our results enlarging the preponderance of Phylum Proteobacteria specifically due to large numbers of *Pseudomonas* sp. reads. Shrike 1412-82980 contributed over 87% of reads belonging to this phylum with most corresponding to *Pseudomonas* sp. and *Diplorickettsia* sp.. This shrike exhibited no clinical signs of illness but, perhaps along with a number of *Rickettsiella* reads may have been impacted by a high ectoparasite load (Figure 3 and Table 1). This latter genus is generally thought of as a pathogen of arthropods, especially ticks (Bouchon et al. 2011). The high number of reads of this genus, along with the high number of *Pseudomonas* sp. reads makes us wonder if this bird carried ticks (*Ixodes* sp. perhaps) and may have experienced such an opportunistic secondary infection (Abd El-Ghany 2021). It is noteworthy that nestling fecal microbiomes increased in *Rickettsiella* abundance over the nestling period in a study of Gray-Backed Shrikes *Lanius tephronotus* (Hu et al. 2022). Also, in that same study, phyla Proteobacteria and Firmicutes predominated. Do shrikes depart from the usual passerine microbiome?

In removing the aforementioned shrike, Phylum Proteobacteria proportional representation falls to 57.4% while the other phyla correspondingly increased (Firmicutes 39.6%, Actinobacteriota 2.4%). Desulfobacterota decreased to 0.1%. Clearly, this individual, showing a substantially different taxonomic distribution than the other shrikes, was experiencing an unusual gut microbiome. Diet and environment have been shown to strongly influence gut microbiome (Teyssier et al. 2018) so we may not even need to rely upon alimentary disfunction in describing this situation. More study on characterizing individual microbiomes needs done.

We observed no statistically significant differences in patterns of OTU (species) evenness and diversity across Northern Shrikes by sex and age (Figures 6-11). This is not surprising in that we only sequenced 19 birds. However, even with this low sample size, a slight trend was seen in which males exhibited higher evenness and diversity values, albeit not significantly so. We can only imagine that if we were to be able to adequately assign sex to all shrikes in hand (i.e., genetically), we may have more readily observed a pattern. Hence, more study is certainly needed here with the call to couple microbiome data with genetic sexing of shrikes for a more comprehensive approach. For instance, the slightly higher values for males may indicate sex differences in microbiome metabolic function rather than evenness and diversity (see Teyssieret al. 2018). Again, our outlying male shrike may be driving our seemingly observed pattern, which may be especially the case in the interpretation of Shannon Entropy, an apparent high amount of disorder or unpredictability in the male shrikes. On the other hand, Herder et al. (2023) note that alpha diversity of the microbiome varies so substantially across bird species, as well as mean body size and season, that significant differences may be spurious.

In conclusion, we show a relatively diverse assemblage of bacteria contained within the feces of wintering Northern Shrikes. A majority of these taxa are associated with terrestrial and/or aquatic environments in a not-surprising pattern following shrike habitat associations and dietary breadth. As shrikes differ substantially in diet from many other passerines, we urge other shrike researchers to collect fecal samples and characterize the microbiome of shrikes encountered. Even though Kropáčková, et al. (2017) found that host ecology has limited effect on the microbiome, host phylogeny and microbiome were intimately linked. This association points to opportunities to describe the microbiome assemblage across the Laniidae and across geography, biome, and diet.

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