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RESEARCH ARTICLE

Picky eaters: Selective microbial diet of avian ectosymbionts

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Abstract

1. Individual organisms can function as ecosystems inhabited by symbionts. Symbionts may interact with each other in ways that subsequently influence their hosts positively or negatively, although the details of how these interactions operate collectively are usually not well understood.
2. Vane-dwelling feather mites are common ectosymbionts of birds and are proposed to confer benefits to hosts by consuming feather-degrading microbes. However, it is unknown whether these mites exhibit generalist or selective diets, or how their dietary selection could potentially impact their symbiotic functional nature.
3. In this study, we conducted 16S rDNA and ITS1 amplicon sequencing to examine the microbial diet of feather mites. We characterized and compared the diversity and composition of bacteria and fungi in the bodies of mites living on feathers of the Prothonotary Warbler, *Protonotaria citrea*, to microbial assemblages present on the same feathers.
4. We found less diverse, more compositionally similar microbial assemblages within mites than on feathers. We also found that mites were resource-selective. Based on the identity and known functions of microbes found within and presumably preferred by mites, our results suggest that these mites selectively consume feather-degrading microbes. Therefore, our results support the proposition that mites confer benefits to their hosts.
5. This study provides insight into symbioses operating at multiple biological levels, highlights the ecological and evolutionary importance of the synergistic interactions between species, and greatly expands our understanding of feather mite biology.

KEYWORDS
Astigmata, birds, feather mites, feather-associated microbes, microbiome, resource selection

1 | INTRODUCTION

Individual organisms can serve as ecosystems of biodiverse symbionts representing all domains of life and non-living biological entities such as prions and viruses (Larsen et al., 2017; Moya et al., 2008). Symbionts (i.e. organisms that live on or within hosts; De Bary, 1879; Hopkins et al., 2017) are ubiquitous across the tree of life and range from mutualistic prokaryotic endosymbionts such as *Buchnera* that provide insect hosts with essential nutrients (Moran et al., 2008) to parasitic eukaryotic ectosymbionts such as blood-feeding lice (Light et al., 2010). Importantly, multiple symbionts may act synergistically to influence hosts either positively (Currie et al., 1999) or negatively (DeCandia et al., 2020). These and other recent studies demonstrate that microbes can influence the relationship between hosts and eukaryotic symbionts (Coolen et al., 2022; Hopkins et al., 2017; Poulin et al., 2023). For example, chemicals produced by skin-associated microbes may help hematophagous ectoparasitic bat flies locate and infect hosts (Lutz et al., 2022), and endosymbiotic bacteria may help leaf-miners manipulate host plant physiology to increase fitness (Kaiser et al., 2010). However, for many hosts and eukaryotic symbionts, the role that microbes play in mediating their interactions remains elusive and presents a major challenge in ecology and evolutionary biology (Dheilly et al., 2019; Hodžić et al., 2023).

Birds harbour a variety of internal and external symbionts and are thus an ideal system to study synergistic interactions between hosts, eukaryotic symbionts, and microbes (Lutz et al., 2017). Some of the most diverse eukaryotic avian symbionts are oribatid mites (Acariformes: Sarcoptiformes: Oribatida). Within Oribatida, the astigmatid mites (cohort Astigmata) comprise some of the most common, yet least understood, avian symbionts. One group of astigmatid mites (parvorder Psoroptidia) is associated with the skin, respiratory passages, and feathers of birds (Proctor, 2003). Feather-associated psoroptids (Analgoidea and Pterolichoidea) occupy different microhabitats, including within and on top of the skin, inside the quills, and on the surface of feathers (Dabert & Mironov, 1999). Most ecological research has focused on feather mites that dwell on the vanes of flight feathers due to the relative ease of observing them on live birds. However, despite their ubiquity in almost every avian order (Doña et al., 2016), specialized adaptations to reside permanently on feathers (Gaud & Atyeo, 1996), and obligate reliance on hosts for survival and transmission (Doña et al., 2017), relatively little is known about the basic biology of feather vane-dwelling mites (e.g. behaviour, diet, reproduction; Proctor, 2003). This gap limits our understanding of their functional relationship with hosts, which has been debated for decades. The literature on this subject suggests that feather mites affect host fitness-related traits such as feather integrity and coloration in both positive (mutualistic; Galván & Sanz, 2006) and negative (parasitic; Figuerola et al., 2003) directions. However, in all cases, a mechanistic understanding of how these impacts arise is not well known. Thus, gaining a better understanding of basic aspects of feather vane-dwelling mite biology and their adaptations to the feather ecosystem could help clarify the uncertainty regarding their effect on hosts.

Diet is a fundamental aspect of an organism's biology that can shape its fitness and survival. With feather mites, their diet could have a direct link to their effect on hosts, yet this link is poorly understood. Members of the Oribatida, including Astigmata, are unique among arachnids because they feed on particulate matter rather than fluids (Walter & Proctor, 2013). This feeding behaviour thereby increases the probability of detecting DNA from prey items in the guts of astigmatan mites. DNA evidence has shown that vane-dwelling feather mites do not consume host tissues like feathers, blood, or skin (Doña et al., 2019), suggesting that they are not objectively harmful. Instead, they primarily consume bacteria and fungi (and possibly uropygial oil) that also reside on feathers (Doña et al., 2019); whether mites functionally operate as microbial predators or grazers within the feather ecosystem is not yet understood, but this concept is an emerging theme in microbial ecology (de la Cruz Barron et al., 2023; Guo et al., 2023; Potapov et al., 2022). Feather mites and other members of the Astigmata exhibit a nutritionally broad diet and are classified as predators, fungivores, microbivores, and/or detritivores (Doña et al., 2019; Walter & Proctor, 2013). Although some acariform mites (e.g. syringophilid quill mites, stored product mites) harbour internal microbes that are likely endosymbiotic (Glowska et al., 2020; Hubert et al., 2021), there is strong evidence that many microbes within Astigmata (e.g. feather mites, synanthropic mites) have originated via consumption and are not all endosymbiotic (Doña et al., 2019; Erban & Hubert, 2008; Hubert et al., 2012; Hubert, Kopecký, et al., 2016). Consequently, it has long been speculated that feather mites provide a 'cleaning service' to birds by removing microbes from feathers (Blanco et al., 2001; OConnor, 1982). This service has been deemed beneficial (i.e. mutualistic) because DNA sequences of harmful microbes (e.g. keratin-degrading) have been detected within the bodies of mites (Doña et al., 2019; Labrador et al., 2022). However, no studies have compared mite dietary composition to the potentially available resources on feathers, which leaves important ecological details about their diet and signatures of foraging behaviour (i.e. whether they prefer or avoid various resources) completely unknown. Further, as is expected for a density-dependent factor like food, dietary preferences, and associated dietary niche breadth may be indirectly driven by the number of competitors (i.e. mite infrapopulation size; Morris, 2003). For example, mites may prefer to consume certain microbes, but this preference may break down above a critical infrapopulation size; above this threshold, competition could cause a shortage in preferred resources and force mites to shift consumption to more abundant, but normally avoided, resources. However, it is unknown if signatures of density-dependent selection exist in this system or if these traits alter the symbiosis between mites and avian hosts.

Here, we address major knowledge gaps regarding basic feather mite biology (i.e. dietary selection) and the role of mites within the feather ecosystem. Specifically, we characterized and compared the bacteria and fungi inside feather mites (consumed resources) to those on the feathers on which the mites reside (potentially available resources). We also counted the number of individuals residing on flight feathers to quantify mite infrapopulation size. Our main

objectives were to measure differences in (1) microbial diversity and composition, and (2) the relative abundance of specific microbial taxa within mites versus on feathers to infer mite dietary and density-dependent selection. If mites exhibit differential microbial diversity, composition, and abundance compared with feathers, then this would represent evidence for a selective diet. Alternatively, if there is no difference in microbial diversity, composition, and abundance then this would represent evidence of a generalist diet. We also identified if microbes found within mites versus on feathers had keratin-degrading properties to determine how mite dietary selection could mediate their functional role on hosts. If mites preferentially consume feather-degrading or pathogenic microbes, this may represent evidence to support a mutualistic relationship that reduces harmful microbes and improves feather quality. Alternatively, if mites avoid feather-degrading microbes, this may represent evidence to support a more parasitic relationship that diminishes feather quality, or an otherwise non-mutualistic relationship. Analysing these complementary microbial assemblages allowed us to more comprehensively study feather mite diet, and for the first time, their resource selection. In doing so, we gained a broader perspective of the role microbes play in the symbiotic relationship between feather mites and hosts.

2 | MATERIALS AND METHODS

2.1 | Study species and sample collection

Prothonotary Warblers (*Protonotaria citrea*) were chosen as the focal host for two primary reasons: their habitat (flooded bottomland hardwood forests) and nesting strategy (secondary cavity nesters; Petit, 2020). We expected that these warm and humid conditions would make Prothonotary Warblers an ideal host for studying the relationships between birds, feather mites, and microbes, as warm and humid environments can increase mite survival and densities on hosts (Gaede & Knülle, 1987; Meléndez et al., 2014), and these hosts serve as a natural 'Petri dish' for microbes under these environmental conditions. We captured birds at Earl Buss Bayou DeView Wildlife Management Area in northeastern Arkansas, USA (35.623 N, 90.943 W) between 30 April 2021 and 14 May 2021, coinciding with the nest building or egg stage of birds' first clutch to precede vertical transmission of mites to nestlings. We used mist-nets and playback to capture 15 territorial adult males, and hand-nets to capture 15 females at pre-installed nest boxes. We removed birds from nets using sterile, single-use latex gloves and immediately collected the third innermost rectrix (i.e. 'R3' tail feather) and placed it in a sterile vial on ice. Mites from this feather, if present, were later censused to quantify a 'feather-level' infrapopulation size (see below). We banded individuals with uniquely numbered United States Geological Survey aluminium bands. We extended the remaining feathers and took macro-lens photos of the ventral side of all flight feathers (wing and tail) to quantify vane-dwelling mites (Matthews et al., 2018). Photos were used to census the remaining mites and this value was added to the 'feather-level' infrapopulation size to calculate a 'host-level' infrapopulation size. We transferred vials containing feathers to a −20°C freezer the same day. Capturing and handling of birds was conducted under USGS permit #23877 and Arkansas State University IACUC #63–8363.

2.2 | DNA isolation and sequencing

DNA from whole mites and feathers was isolated using the Qiagen DNeasy PowerSoil Pro Kit (Cat. #47014) under sterile conditions. We used forceps to count ('feather-level' infrapopulation) and place mites into a glass dish containing 70% ethanol. Of the 30 feathers we sampled, 29 harboured mites. Of these 29 feathers, 25 (86%) harboured predominately *Amerodectes protonotaria* Hernandes 2018 (Analgoidea: Proctophyllodidae: Pterodectinae), which is a Prothonotary Warbler-specialist mite (Matthews et al., 2023). On average, there were 54.4 ± 12.3 (SE; SD = 67.6) *A. protonotaria* mites per feather. Of those 25, eight (32%) also harboured <5 individual mites of the confamilial genus *Proctophyllodes* (Analgoidea: Proctophyllodidae: Proctophyllodinae). The remaining four feathers (14%) harboured <7 *Proctophyllodes* individuals. Heterogeneric mites were not separated for DNA isolation (i.e. all mites from a single feather were extracted together) because both species are members of the family Proctophyllodidae, Doña et al. (2019) found that microbial diversity and composition did not differ greatly between pterodectines and proctophyllodines, and they differed in abundance in our samples. Mites were surface-sterilized with three 70% ethanol washes (Andrews, 2013). After all ethanol evaporated following the third wash, we transferred mites to a bead tube containing CD1 solution (lysis buffer). We then cut the mite-free feather vane into small pieces and transferred the pieces into a bead tube containing CD1. In total, we processed 30 feather samples, 29 pools of mites, and several negative controls to account for contaminants in low-biomass samples and commercial extraction kits (described below).

For mites from five birds, leftover ethanol from the third sterilizing wash was used as a negative control and was pipetted onto nitrocellulose paper (BioRad Cat. #1620112; pore size 0.2 µm, 2.5cm^2) until saturated. After the paper dried completely, we cut it into pieces small enough to fit into the bead tube containing CD1. The same method was used for one 100% ethanol and one nuclease-free water control. We also included one control for each day we conducted extractions, in which the bead tube containing CD1 was left open in the area during extractions ($n = 4$). Lastly, we included two controls for each of the two extraction kits used ($n = 4$) in which 'blanks' (i.e. no samples added) were extracted to account for microbial contamination found in commercial extraction kits (Salter et al., 2014).

For bacteria, we targeted the V4 region of the 16S ribosomal RNA (rRNA) gene (515F/806R; Caporaso et al., 2012), and for fungi, we targeted the internal transcribed spacer 1 (ITS1) region (ITS1F/ITS2R; White et al., 1990). Library preparation, purification, and

Illumina sequencing (16S: MiniSeq 2x153bp; ITS1: MiSeq 2x300bp) were performed at the DNA Services facility, Research Resources Center, University of Illinois (see Supporting Information for detailed molecular protocols).

2.3 | Bioinformatic analyses

For 16S bacterial sequences ($n = 76$ samples), a total of 2,845,702 raw Illumina reads were trimmed (using the cutadapt [Martin, 2011] plugin), paired, and quality filtered using DADA2's 'denoise-paired' pipeline (Callahan et al., 2016) in QIIME2 version 2022.2 (Bolyen et al., 2019). We truncated reverse reads to 130 bp, truncated reads at the first instance of a quality score ≤ 15, changed the minimum length of overlap for merging forward and reverse reads to 4, and assigned the minimum fold-change parent sequence over abundance to 4. Reads were denoised into 4885 amplicon sequence variants (ASVs), which were identified using SILVA release 138 (Quast et al., 2013). ASVs were filtered to exclude sequences of non-bacterial origin (archaea, eukaryotes, mitochondria, and plant chloroplasts), reducing the dataset to 826,580 total reads and 4253 ASVs. We used 'decontam' version 1.16.0 (Davis et al., 2018) to identify and remove potential contaminant ASVs using a prevalence threshold of 0.5, which retained 621,094 reads and 4064 ASVs. We removed control samples and samples with fewer than 500 reads (which retained 41 samples and 2834 ASVs; Table S1) before analysing diversity metrics in QIIME2. We used this reduced suite of bacterial ASVs for taxonomy composition and other downstream analyses.

For ITS1 fungal sequences ($n = 76$ samples), we used the forward reads for analyses because (1) forward reads were of much higher quality than reverse reads, and (2) more reads were lost or were of insufficient quality after merging forward and reverse reads because of a lack of sufficient overlap (Hoggard et al., 2018). A total of 1,498,216 raw forward Illumina reads were trimmed and quality filtered using DADA2's 'denoise-single' in QIIME2. We used all default denoising parameters except that we truncated reads to 275 bp and truncated reads at the first instance of a quality score ≤ 15. Reads were denoised into 4724 ASVs, which were identified using UNITE (version 9, release date 29.11.2022; Kõljalg et al., 2020; Nilsson et al., 2019). No ASVs were identified as non-fungal. We decontaminated sequences as we did for 16S, resulting in 474,534 total reads and 4685 ASVs. We removed control samples and samples with fewer than 500 reads (which retained 48 samples and 4461 ASVs; Table S2) before analysing diversity metrics in QIIME2. We used this reduced suite of fungal ASVs for taxonomy composition and other downstream analyses.

2.4 | Statistical analyses

To measure differences in microbial diversity at the ASV-level, we estimated within-sample diversity (alpha diversity) of microbes in mites and on feathers using Shannon and Chao1 indices. The Shannon index is a quantitative measure of the number of ASVs (richness) and their relative abundance (evenness), whereas Chao1 is a quantitative measure of ASV richness only. Higher values indicate higher diversity. We performed Kruskal–Wallis tests with a false discovery rate (FDR) correction in QIIME2 to assess differences in bacterial ($n = 41$) and fungal ($n = 48$) alpha diversity indices between all mites and all feathers. We also performed a Wilcoxon signed-rank test on the subset of samples that were paired by individual host in the bacterial ($n = 32$ out of 41; 16 pairs) and fungal ($n = 40$ out of 48; 20 pairs) datasets in R version 4.2.3 (R Core Team, 2023).

To measure differences in microbial composition at the ASV-level (i.e. membership and structure; beta-diversity) between mites and feathers, we employed a compositional approach using Robust Aitchison Principal Component Analysis (RPCA) with the Gemelli QIIME2 plugin (Martino et al., 2019). All samples were included in these analyses (bacteria: $n = 41$; fungi: $n = 48$). Permutational multivariate analysis of variance (PERMANOVA; 999 permutations) was performed to analyse whether mite microbial beta-diversity differed significantly from that of feathers. We used permutational analysis of dispersion (PERMDISP; 999 permutations) to test the homogeneity of multivariate dispersions within groups (mites and feathers).

To measure differences in the relative abundance of specific bacterial and fungal taxa (at the phylum, family, and genus levels) within mites versus on feathers, we used 'MaAsLin2' version 1.10.0 (Mallick et al., 2021) in R. All samples were included in differential abundance (DA) analyses (bacteria: $n = 41$; fungi: $n = 48$). We specified the CPLM (Compound Poisson [generalized] Linear Model) analysis method, the minimum prevalence threshold was left at the default (0.1), and we specified 'none' for normalization because relative abundance data were used as input. We included the individual bird as a random effect and applied FDR corrections. For all analyses, we considered p-values and FDR-adjusted p-values (i.e. Q-values) ≤ 0.05 as significant.

To infer whether mites were selected for or against particular potentially available microbial resources at the genus level, we calculated Vanderploeg and Scavia's relativized electivity index (Lechowicz, 1982; Vanderploeg & Scavia, 1979). This index was calculated from relative abundance data using 'dietr' version 1.1.4 (Borstein, 2020) in R. This index ($E*$) is similar to a normalized foraging ratio (W_i):

$$E_i^* = \frac{\left[W_i - \left(\frac{1}{n}\right)\right]}{\left[W_i + \left(\frac{1}{n}\right)\right]},$$

where

$$W_i = \frac{\frac{r_i}{p_i}}{\sum_i \left(\frac{r_i}{p_i}\right)}.$$

Here, r_i and p_i represent the relative proportion (i.e. relative abundance) of resource i consumed and available, respectively, and n

represents the number of different types of resources. Calculating E_i^* requires paired data (i.e. consumed versus available), so we only used paired samples (bacteria: $n=16$ pairs; fungi: $n=20$ pairs) to calculate a per sample (i.e. individual bird) electivity index for each potentially available resource. The index ranges from −1 to 1. Values closer to −1 indicate that resource *i* is 'avoided' (proportionally, resource *i* is underrepresented in the diet [mites] compared with its availability in the environment [feather]) whereas values closer to 1 indicate that resource *i* is 'preferred' (resource *i* is proportionally overrepresented in the diet compared with the environment). Values of zero indicate that the proportion of resource *i* is the same in the diet as it is available in the environment (neutral selection; no preference for or against resource *i*). If the 95% confidence intervals (CIs) of the mean E_i^* across all samples did not overlap zero, we considered selection for or against resource *i* significant. If the 95% CIs overlapped zero, we considered the selection for resource *i* random.

We also calculated an overall electivity index per sample by averaging E_i^* over the 10 most abundant microbial genera available to mites ('pickiness score'; $\overline{E*}$). We used the 'pickiness score' as a response variable with (1) the number of mites residing on the feather (mean number of mites ± SE = 55.7 ± 12.3 [SD = 67.4]; range = 0–267 mites), or (2) the total number of mites on each individual host (249 ± 37.9 [207.7]; range = 17–712 mites) as predictors in separate linear models to determine if resource selection is density-dependent at either the feather- or host-level, respectively. If mites select for (i.e., prefer) a greater amount of potentially available resources, $\overline{E*}$ will be closer to 1, indicating they are less 'picky'. If mites select against (i.e., avoid) most potentially available resources, $\overline{E*}$ will be closer to −1, indicating mites are more 'picky'. Linear models were built using a Gaussian error distribution and an identity link function in 'stats' (R Core Team, 2023). To confirm the normality of model residuals and ensure that models fit data well, we performed Shapiro–Wilk tests (Shapiro & Wilk, 1965). If the *p*-value was ≥0.05, the residuals did not significantly differ from a normal distribution, and we considered the model valid.

2.5 | Literature search on microbial feather-degrading functions

We conducted a literature search using the Web of Science database to identify potential feather-degrading functions (e.g. keratinolytic activity or production of keratinase) of significantly differentially abundant microbes, the 10 most abundant microbial genera available to mites, and the genera mites selected for based on electivity analyses. To capture the most relevant publications, we used quotation marks around each genus name along with the Boolean operator 'AND' alongside the term 'keratin*'. The asterisk '*' served as a truncation operator to include related terms such as 'keratinase' or 'keratinolytic'. We reviewed up to the top 10 most relevant articles to ascertain the potential feather-degrading function for each microbial genus.

3 | RESULTS

3.1 | Feathers and mites harboured distinct bacterial assemblages

Bacterial assemblages within mites were significantly less diverse than bacterial assemblages on feathers. Shannon diversity was significantly lower within mites (3.47 ± 0.27) than on feathers (5.14 ± 0.22; $H=13.14$, $p<0.001$; Figure 1a), as was Chao1 (mites: 45.6 ± 9.1 vs. feathers: 138 ± 15.7; $H=15.62$, $p<0.001$; Figure 1c). Furthermore, in 15 out of 16 paired samples (i.e. birds), Shannon diversity was lower in mites (3.1 ± 0.24) compared with feathers (5.18 ± 0.32; $V=134$, $p<0.001$). In 14 out of 16 pairs, Chao1 was lower in mites (34.9 ± 3.35) than feathers (155 ± 21.5; $V=133$, $p<0.001$). Beta-diversity also differed significantly between mites and feathers (PERMANOVA pseudo-$F=7.72$, $p=0.001$; Figure 2a). We observed reduced inter-individual variation among mite bacterial assemblages compared with those of feathers (PERMDISP $F=22.06$, $p=0.001$; Figure 2a), indicating that mite bacterial microbes are more compositionally homogeneous than those of feathers.

From a total of 2834 bacterial ASVs, we identified 33 different phyla on feathers and 20 phyla within mites (Figure 3a, Figure S1A). Dominant phyla included Proteobacteria (mites: 69.3% ± 5.0%, feathers: 48.1% ± 4.7%), Firmicutes (mites: 15.9% ± 3.9%, feathers: 10.5% ± 3.2%), and Bacteroidota (mites: 8.8% ± 1.6%, feathers: 10.8% ± 1.8%). Feathers had a high average relative abundance of Actinobacteriota (20.7% ± 4.9%), whereas mites did not (1.8% ± 0.4%).

Relative abundance of bacteria differed significantly ($Q\leq0.05$) between mites and feathers for eight phyla, 26 families, and 18 genera based on DA analyses (Figure 4a,b; Tables S3 and S4). Our literature search consisted of 44 bacterial genera; seven were found to exhibit keratin-degrading properties, with four of those seven having a greater average relative abundance in mites than on feathers, and two of those four being significantly differentially abundant (Table S3). Overall, five of the 18 differentially abundant genera have known keratin-degrading properties (Table S3). Based on electivity analyses, mites selected for (preferred) eight bacterial genera (three of which are known to exhibit keratin-degrading properties), selected against (avoided) 478 bacterial genera, and exhibited no selection for or against 13 bacterial genera (Figure 5a; Tables S3 and S4). The overall average 'pickiness score' was −0.38 ± 0.05 [SE]; this negative value indicates that mites selected against most potentially available resources. The number of mites present on a feather was not a significant predictor of average bacterial selectivity ($t_{14}=1.25$, $p=0.23$). However, there was a nearly significant positive correlation between the number of mites present across the entire host and the average bacterial selectivity ($t_{14}=1.93$, $p=0.07$; Figure S2). The correlation in this case indicates that as the number of mites increases across the host, they select for (rather than against) a greater amount of potentially available bacterial resources (i.e. they are less 'picky').

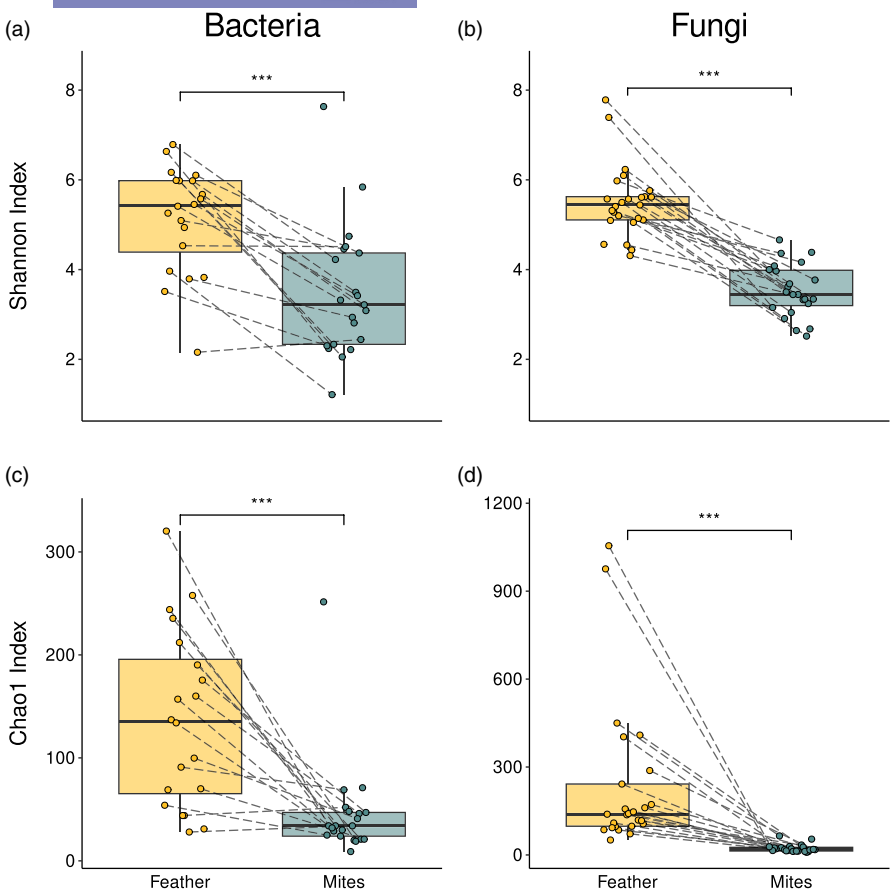

FIGURE 1 Shannon (a, b) and Chao1 (c, d) alpha diversity indices differed between bacterial (a, c) and fungal (b, d) assemblages on feathers (gold, left boxplots) and within mites (teal, right boxplots). *** denotes $p < 0.001$ after FDR correction. Each point represents a sample and dashed lines connecting points represent paired samples (i.e. microbes from mites and the exact feather from which the mites were removed).

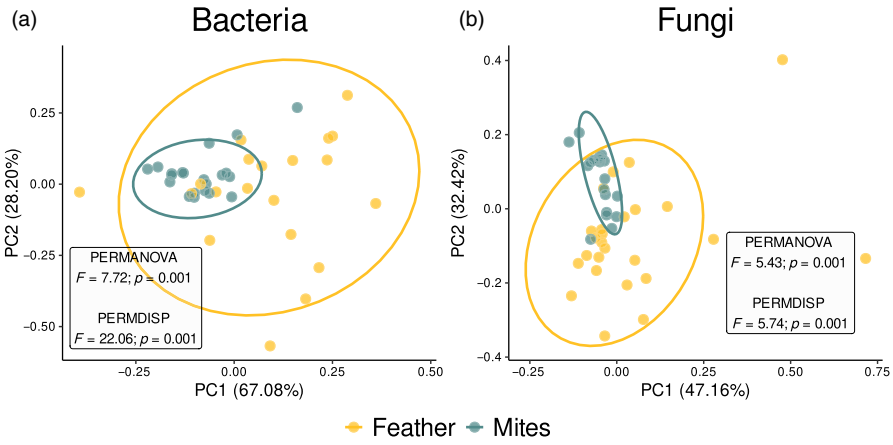

FIGURE 2 Robust Aitchison Principal Coordinates Analysis illustrating significant differences in bacterial (a) and fungal (b) structure between feathers (gold) and mites (teal). Ellipses are drawn at a 95% confidence level.

3.2 | Feathers and mites harboured distinct fungal assemblages

Fungal assemblages within mites were significantly less diverse than those on feathers. Shannon diversity was significantly lower within mites (3.53 ± 0.11) than on feathers (5.51 ± 0.15; $H = 33.75$, $p < 0.001$; Figure 1b), as was Chao1 (mites: 22.3 ± 2.47 versus feathers: 236 ± 47.2; $H = 34.73$, $p < 0.001$; Figure 1d). Furthermore, in all 20 paired samples (i.e. birds), Shannon diversity was lower within mites (3.54 ± 0.13) compared with feathers (5.63 ± 0.18; $V = 210$, $p < 0.001$), as was Chao1 (mites: 21.5 ± 2.76 versus feathers: 272 ± 62.3; $V = 210$, $p < 0.001$). Beta-diversity also differed significantly between mites and feathers (PERMANOVA pseudo-$F = 5.43$, $p = 0.001$; Figure 2b). We observed reduced inter-individual variation among mite fungal assemblages compared with those of feathers (PERMDISP $F = 5.74$, $p = 0.001$; Figure 2b), indicating that mite fungal microbes are more compositionally homogeneous than those of feathers.

From a total of 4461 fungal ASVs, we detected six phyla in feathers and four phyla in mites (Figure 3b, Figure S1B), dominated by Ascomycota (mites: 73.1% ± 3.6%, feathers: 87.3% ± 1.6%) and Basidiomycota (mites: 19.5% ± 2.9%, feathers: 6% ± 1.5%). Based

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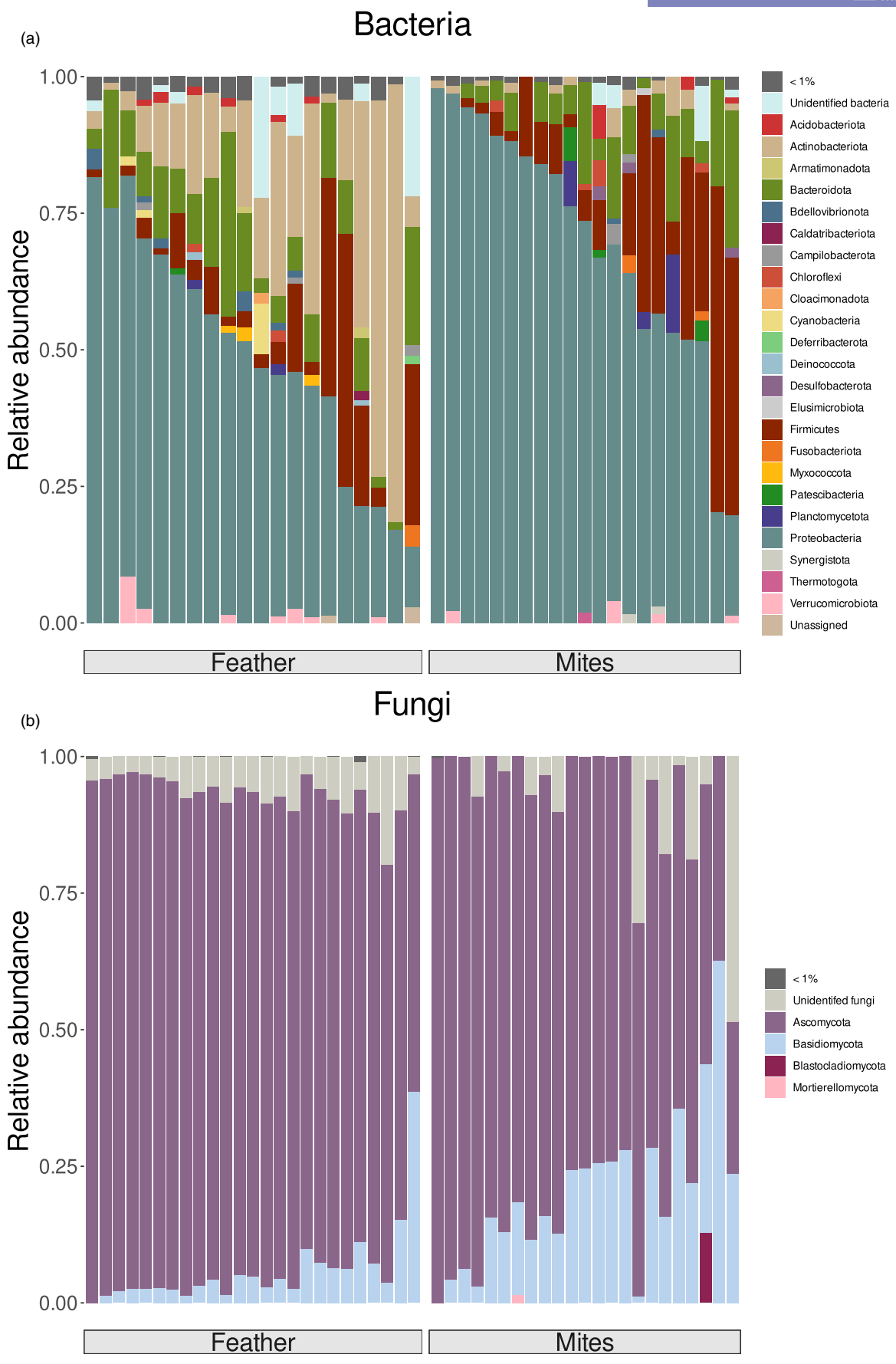

FIGURE 3 Stacked bar plots depicting the relative abundances of bacterial (a) and fungal (b) phyla found on feathers and within mites. Columns represent samples and are ordered by decreasing the relative abundance value of the most abundant phylum. Low abundance taxa (<1%) are collapsed.

on DA analyses, relative abundance of fungi significantly differed ($Q \leq 0.05$) between mites and feathers for two phyla, 21 families, and 27 genera (Figure 4c,d; Tables S3 and S4). Our literature search consisted of 61 fungal genera; 14 were found to exhibit keratin-degrading properties, with 11 of those 14 having a greater average relative abundance in mites than on feathers, and five of those 11 being significantly differentially abundant (Table S3). Overall, eight of the 27 differentially abundant genera have known keratin-degrading properties (Table S3). Based on electivity analyses, mites selected for two fungal genera (neither of which are known to exhibit keratin-degrading properties), selected against 520 fungal genera, and exhibited no selection for or against 13 fungal genera (Figure 5b; Tables S3 and S4). The average 'pickiness score' was -0.64 ± 0.05 [SE] for fungi. Neither the number of mites present on a feather ($t_{18} = 0.5$, $p = 0.62$) nor the number of mites present across the entire host ($t_{18} = 0.93$, $p = 0.37$) were significant predictors of average mite fungal selectivity, indicating mites' selection of fungi is not density-dependent.

4 | DISCUSSION

In this study, we demonstrated that vane-dwelling feather mites and the feathers on which they live differ in microbial diversity and composition. Specifically, mites exhibit lower diversity, and more compositionally homogeneous assemblages, and the relative abundance of several microbial taxa differs between mites and feathers. Our results also indicate that mites appear to prefer only a few microbes to consume and avoid the majority (i.e. they are 'picky' eaters). In addition, their resource selection pattern does not seem to be strongly affected by infrapopulation sizes at the feather- or host-level, suggesting it is not density-dependent. Based on the known functions of the microbes that mites preferred, it is possible that feather mites are providing a beneficial service to their hosts by removing keratin-degrading microbes from feathers. More broadly, our results reveal new details regarding the role that microbes play in mediating the interactions between hosts and eukaryotic ectosymbionts.

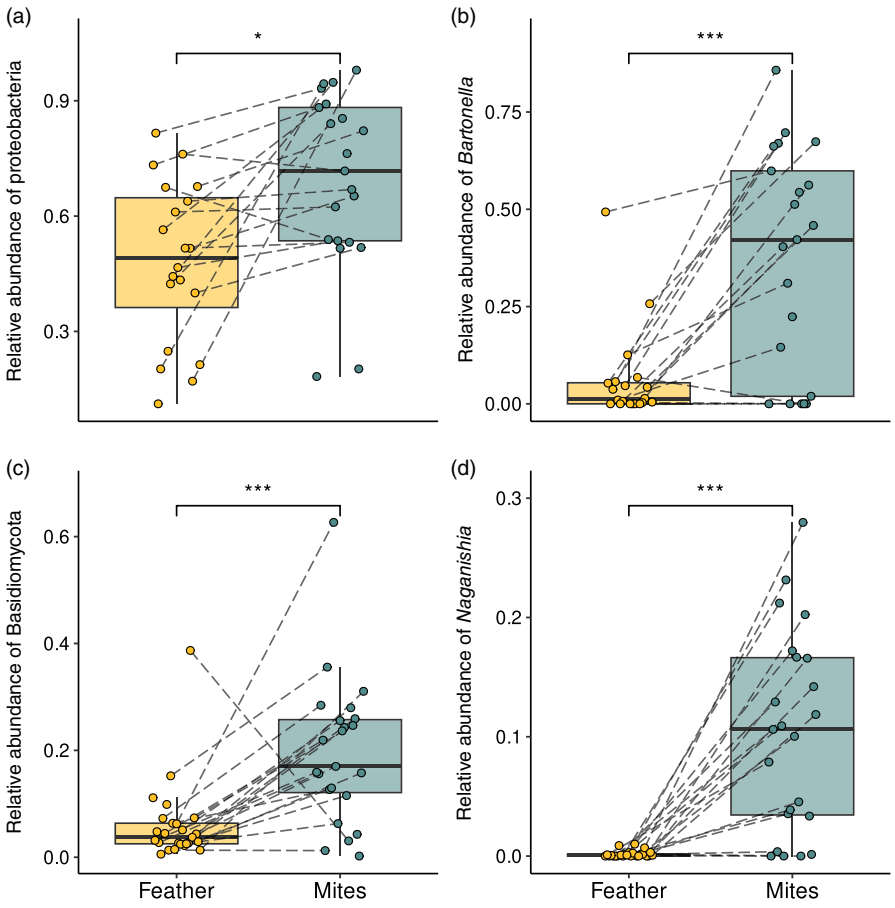

FIGURE 4 Relative abundance of bacteria (a, b) and fungi (c, d) between feathers (left, gold) and mites (right, teal) differed at multiple taxonomic levels (a, c: phylum; b, d: genus). *** denotes $p < 0.001$ and * denotes $p < 0.05$ after FDR correction. Each point represents a sample and dashed lines connecting points represent paired samples (i.e. microbes from mites and the exact feather from which the mites were removed).

FIGURE 5 Vanderploeg and Scavia's Relativized Electivity Index for available (a) bacterial and (b) fungal genera, ordered taxonomically. Horizontal lines indicate 95% confidence intervals around mean electivity for each microbial resource. Positive values (blue) indicate selection for the resource, whereas negative values (red) indicate avoidance of the resource. Electivity that overlaps the vertical dotted line (grey) indicates no selection for or against the resource. Only genera with more than one representative sample of available resources and those with a mean electivity > −1 are illustrated. An asterisk (*) indicates taxa that were also identified as significantly differentially abundant in differential abundance analyses. Taxa followed by the letters 'FD' represent those that have known keratin/feather-degrading functional properties (sources located in Table S3).

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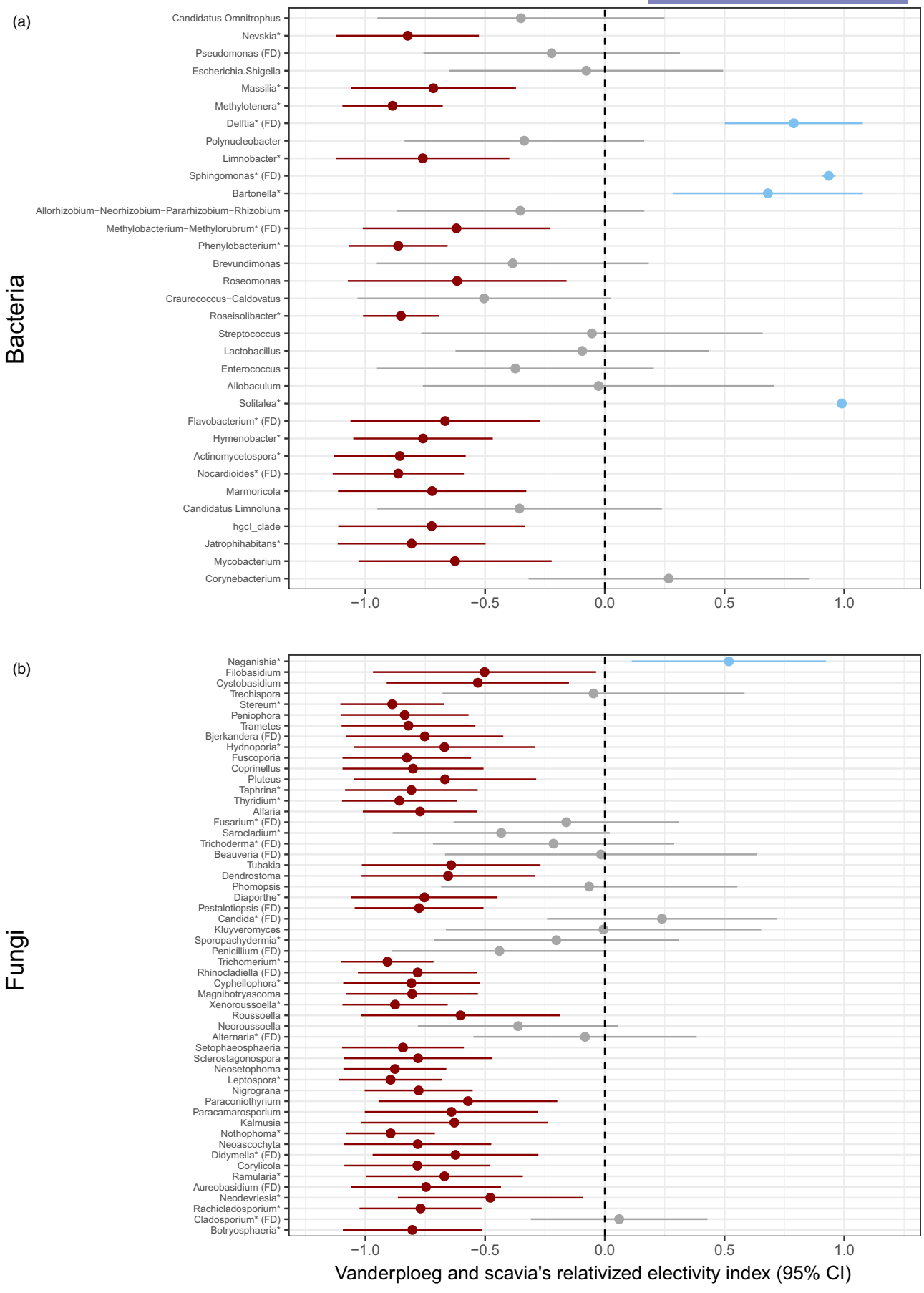

4.1 | Feather mites exhibit a selective microbial diet

Feather mite microbial assemblages were significantly less diverse and were more compositionally homogeneous than those of the feathers where they reside (Figures 1 and 2). This pattern is consistent with other groups of mites; stored product mites and honeybee mites have lower microbial diversity than the seeds and bees on which they live and feed, respectively (Hubert, Kamler, et al., 2016; Hubert et al., 2003). Our findings complement those of Doña et al. (2019), which used light microscopy and DNA metabarcoding to examine the gut contents of multiple feather mite species across a broad range of host species; this large-scale study revealed that mites consume a diverse array of bacterial and fungal taxa (many taxa overlapped in the two studies, see below). In the present study, we compared mite gut contents to the feathers on which the mites were residing. This feather-to-mite comparison allowed us to thus also assess the trophic strategy of feather mites, which appears to be selective. It is indeed possible that mites do not interact with the entire feather-associated microbiome equally for feeding; however, this seems unlikely as some species of mites move along and feed from feathers throughout the day and night (Labrador et al., 2022). Despite these movements, mites fine-tune their spatial distribution across host feathers (Jovani & Serrano, 2004; Stefan et al., 2015) in a somewhat predictable pattern, which may be related to food availability. Future experimental studies may help to identify in which (if any) feather microhabitat mites choose to feed, or if any other host-associated microbiomes drive the availability of resources on feathers (e.g. the uropygial gland [Grieves et al., 2021], cloaca [van Veelen et al., 2017], or gut [Baiz et al., 2023]). Nevertheless, our results indicate that feather mites exhibit a narrow dietary niche consisting mainly of a few microbial genera, and add to the growing knowledge regarding how feather mites interact with feather-associated microbes (Doña et al., 2019; Labrador et al., 2022) and operate within the feather ecosystem more generally (Labrador et al., 2024). Although the taxonomic resolution is limited due to sequencing technology, several microbial genera were highly prevalent in the *Amerodectes* and *Proctophyllodes* mites in our study (Table S3) and in four feather mite genera (including *Proctophyllodes*) studied by Doña et al. (2019). These microbial genera included *Alternaria*, *Aureobasidium*, *Bartonella*, *Cladosporium*, *Escherichia-Shigella*, *Methylobacterium*, *Naganishia*, *Pseudomonas*, and *Sphingomonas*, suggesting that these taxa appear to be favoured by and prevalent in multiple species of feather mites. Several of these same genera have been detected on feathers of other passerines (e.g. *Alternaria*, *Cladosporium*, *Methylobacterium*, and *Sphingomonas*; Dille et al., 2016; Hotopp et al., 2024; Silva et al., 2022; Tran et al., 2022), further illustrating the consistency of these microbes across feather mite host species.

Certainly, because we assessed microbes and resource selection primarily in a single mite–host pair (*Amerodectes protonotaria* and its only known host, the Prothonotary Warbler, though we also sequenced microbes from a much smaller number of *Proctophyllodes* individuals), the patterns observed here may not represent the typical patterns of all host-mite pairs. Given the high specificity of the primary host-mite pair we studied herein, it is possible that we detected higher resource selectivity than we would with a host generalist mite with perhaps a broader dietary niche. Doña et al. (2019) found that feather mites representing a wide range of host specificities exhibit wide microbial gut diversity, though diversity was not structured by host or mite species. Future investigations of how variation in mite host specificity may relate to microbial diversity and dietary selectivity are warranted and may reveal host- or symbiont-specific traits that could explain the unexpectedly wide range of host specificity in feather mites (Matthews et al., 2023).

4.2 | Feather mite diet supports potential mutualistic relationship with hosts

Among the 44 bacterial genera on which we conducted a literature search, seven are known to exhibit the ability to degrade keratin (Table S3). Three of these seven were inferred as preferred by mites (*Aeromonas*, *Delftia*, and *Sphingomonas*), three were inferred as avoided (*Flavobacterium*, *Methylobacterium–Methylorubrum*, and *Nocardioides*), and mites were neutral towards one (*Pseudomonas*). Of those seven, *Delftia* and *Sphingomonas* were the two most prevalent bacterial genera among mite samples (as well as all samples combined), whereas all others were present in less than 34% of mite samples (Figure 5a; Table S3). The preferential consumption of these two highly prevalent keratin-degrading genera, as well as of the third most prevalent bacteria, *Bartonella*, suggests that mites' diet and selection of microbes may influence the functional nature of the symbiosis. *Delftia* and *Sphingomonas* exhibit feather-degrading (e.g. keratinolytic) activity (Herzog et al., 2016; Tran et al., 2022), and *Bartonella* are gram-negative bacteria typically transmitted by hematophagous arthropods (a trophic behaviour that vane-dwelling feather mites do not exhibit; Doña et al., 2019; Proctor, 2003) that can cause infections in vertebrates, including birds (Mascarelli et al., 2014). Thus, the preferential consumption and removal of these bacteria by feather mites would benefit the host (i.e. mites represent 'cleaning mutualists'), which supports results from Doña et al. (2019). Nevertheless, many bacteria have no known keratin-degrading abilities (Table S3), and whether this is due to a lack of testing, published literature, or true lack of ability, alternative hypotheses should be tested in an experimental and functional context (e.g. keratinase assays, experimental dietary choice assays, functional metagenomics) to fully understand how microbes can impact the nature of the symbiosis between feather mites and hosts. However, because *Bartonella* has also been considered putative endosymbionts across multiple groups of mites including Astigmata, Prostigmata, and Mesostigmata (Hubert et al., 2021; Kopecký et al., 2014; Osuna-Mascaró et al., 2021), and because *Sphingomonas* is prevalent within the bodies of many different mite taxa (Doña et al., 2019; Glowska et al., 2020; Hubert et al., 2021; Osuna-Mascaró et al., 2021), we cannot rule out the possibility that

these organisms are endosymbionts rather than components of the feather mites' diets.

Although we cannot empirically exclude the possibility that some cases of putative preferential resource selectivity by mites are in fact cases of primary endosymbiotic bacteria of mites (as we did not directly observe mite feeding behaviours), several lines of evidence strongly support the interpretation of selective consumption by mites. First, both *Bartonella* and *Sphingomonas* were commonly found on feathers (70% and 95% of feathers, respectively), albeit at lower relative abundances than within mites (Table S3). The high prevalence and low relative abundance of these genera on feathers suggest that they could have originated on feathers and been removed by mites via consumption. *Sphingomonas* has also been found on feathers of other passerines, further suggesting origination on feathers (Silva et al., 2022; Tran et al., 2022). Second, although an endosymbiotic origin could explain the increased relative abundance of some bacteria within mites versus on feathers, it cannot explain the many genera that mites completely avoided (Figure 5). If mites unequivocally select against some resources, they must also select for others. Finally, we found that mites preferred some fungi as well (see below), and to the best of our knowledge, there are no known endosymbiotic fungi of acariform mites. Thus, it seems most plausible that if feather mites are selective in their consumption of fungi, they would also be selective towards bacteria. Overall, this evidence suggests that the patterns uncovered herein are a result of selective consumption and are not due to endosymbiotic microbes. Certainly, more rigorous empirical methods to tease apart these possibilities exist, such as comparing the genome size of internal microbes to free-living microbes (McCutcheon & Moran, 2012), as well as sequencing the full-length 16S rRNA gene or constructing metagenome-assembled bacterial genomes. Further experimental research is also needed to understand the mechanisms underlying why or how mites selectively consume particular microbes. For example, what traits of the mites or microbes (e.g. physiological, chemical, morphological) allow mites to preferentially select for or against certain microbes? Additionally, how do these factors shape microbial composition in the mites? It would be advantageous to estimate absolute microbial quantities and abundances (Labrador et al., 2021), as opposed to relative abundances, to further support whether selection is associated with higher absolute availability.

Among the 61 fungal genera on which we conducted a literature search, 14 have known keratin-degrading functions (11 of which have a higher average relative abundance within mites than on feathers; Table S3). While none of these were inferred as preferred by mites, two of the three most prevalent genera within mites have keratin-degrading functions (*Candida* and *Cladosporium*) as do two of the three most prevalent genera across all samples (*Alternaria and Cladosporium*). Mites preferred *Naganishia* (the other most prevalent genus within mites and across all samples; Figure 4d) and *Dothiorella*, whereas their selection was neutral towards *Alternaria*, *Candida*, and *Cladosporium* (Figure 5b). No keratinase activity has been identified in fungi of the genera *Naganishia* (but see Decostere et al., 2003) or *Dothiorella*, but *Alternaria*, *Candida*, and *Cladosporium* are all known to exhibit keratin-degrading abilities (Călin et al., 2017; Duarte et al., 2011; Marcondes et al., 2008). With *A. protonotaria* exhibiting a neutral selection towards some feather-degrading fungi (and others such as *Beauveria*, *Fusarium*, *Penicillium*, and *Trichoderma*), it suggests that they are, to some extent, providing a cleaning service to their hosts by removing these genera, but perhaps not selecting for them at such a rate that hosts are completely free of them.

4.3 | No strong support for density-dependent dietary selection

Electivity for resources was expected to be density-dependent given that the availability of dietary resources can be limited by mite infrapopulation size and can lead to competition for those limited resources. Our results did not strongly support this hypothesis for either bacteria or fungi. Consequently, the density of mites is not likely a major factor to their dietary selection. However, we uncovered a nearly significant positive relationship between selection for bacteria and the 'host-level' (i.e. total) mite infrapopulation size (Figure S2). Mites are not restricted to individual feathers and can disperse quickly (Matthews et al., 2022), with substantial movements occurring at night in some species when they are most likely feeding (Labrador et al., 2022). Thus, despite the lack of statistical significance, our results indicate that there is potential for density-dependent resource selection and that competition for (bacterial) resources possibly operates at the level of the host. Exploring this relationship in an experimental context in which the number of mites and the amount of food resources can be controlled (Cebolla et al., 2009) may help elucidate if density-dependent resource selection is occurring or if it is context-dependent. The ability to culture feather mites, off-host in vitro, is a necessary first step to isolating these factors, and is one of the many grand challenges in feather mite biology (Proctor, 2022).

5 | CONCLUSIONS

Overall, we demonstrate that the microbial assemblage found within mites is less diverse and more compositionally homogeneous than that of the feathers on which they live. DA analyses and electivity tests suggest that feather mites of Prothonotary Warblers selectively consume particular microbes on feathers (i.e. these mites are 'picky' eaters). Based on the known functions of the preferred microbes, our results further support the idea that these mites could be providing a mutualistic cleaning service to their hosts by removing feather-degrading microbes (Blanco et al., 2001; Doña et al., 2019). Accordingly, it is important to protect the critical link feather mites maintain between their avian hosts and feather-associated microbes (Speer et al., 2020; Sullivan & Ozman-Sullivan, 2021). The generality of these results requires further investigation, and future studies are warranted to determine how mites selectively consume certain microbes and to quantify the subsequent impact of the mites'

service on host fitness. Overall, our study not only greatly expands our understanding of basic feather mite biology and their associated microbes, but also contributes to a broader understanding of how microbes can mediate the relationship between hosts and eukaryotic symbionts. Lastly, our study highlights the potential for future investigations to examine the multitude of interactions within animal ecosystems to discover more about the ecology and evolution of symbioses.

AUTHOR CONTRIBUTIONS

Alix E. Matthews and Than J. Boves conceived the study and designed methodology with contributions from Brian K. Trevelline and Asela J. Wijeratne, ensuring a diversity of perspectives from project onset; Alix E. Matthews secured funding; Alix E. Matthews and Than J. Boves conducted field work and collected samples within the Arkansas Delta region, where three of the authors live; Alix E. Matthews performed molecular work, analysed data, and conducted statistical analyses with input from Brian K. Trevelline and Asela J. Wijeratne; Alix E. Matthews led the writing of the manuscript. All authors were involved in constructively editing drafts and gave approval for publication.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

Raw sequence data were submitted to the Sequence Read Archive at the National Center for Biotechnology Information (BioProject PRJNA1018522). Bioinformatic pipelines and codes for analyses are available on GitHub (https://github.com/alixmatthews/Pcitrea_fm_microbes). Data are also available from the Dryad Digital Repository https://doi.org/10.5061/dryad.r2280gbnc (Matthews et al., 2024).

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

Table S1. Final feature (i.e. amplicon sequence variant [ASV]) table for 16S sequences that was used for all downstream analyses.

Table S2. Final feature (i.e. amplicon sequence variant [ASV]) table for ITS sequences that was used for all downstream analyses.

Table S3. Prevalence and relative abundance (mean ± standard error) of significantly differentially abundant bacterial and fungal genera based on differential abundance analyses (indicated by a *), the 10 most abundant microbial genera available to mites (indicated by a ^), microbial genera that mites selected for (indicated by a +), and microbial genera from the electivity analysis that are depicted in Figure 5 (indicated by a #).

Table S4. Results from differential abundance (DA) analyses for bacteria and fungi at the phylum, family, and genus levels.

Figure S1. Stacked bar plots depicting the relative abundances of bacterial (A) and fungal (B) phyla found on feathers and within mites. Columns represent samples and are ordered by sample

ID. Paired samples come from the same individual bird and have matching identification numbers. For example, PF01 refers to the feather from "Bird 01," and PM01 refers to the mites that were collected from that feather. Low abundance taxa (<1%) are collapsed.

Figure S2. Marginally significant relationship between the number of mites on the entire host and average selectivity based on the 10 most available bacterial resources and associated Vanderploeg and Scavia's Relativized Electivity Indices ($t_{14}=1.93$, $p=0.07$). Electivity values below the horizontal red dotted line represent bacteria avoidance (i.e., on average, most available bacteria are selected against/avoided) and values above the line represent bacteria preference (i.e., on average, most available bacteria are selected for/preferred).

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