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Effects of Host Species Identity and Diet on the Biodiversity of the Microbiota in Puerto Rican Bats

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Abstract

Microbiota perform vital functions for their mammalian hosts, making them potential drivers of host evolution. Understanding effects of environmental factors and host characteristics on the composition and biodiversity of the microbiota may provide novel insights into the origin and maintenance of these symbiotic relationships. Our goals were to (1) characterize biodiversity of oral and rectal microbiota in bats from Puerto Rico; and (2) determine the effects of geographic location and host characteristics on that biodiversity. We collected bats and their microbiota from three sites, and used four metrics (species richness, Shannon diversity, Camargo evenness, Berger-Parker dominance) to characterize biodiversity. We quantified the relative importance of site, host sex, host species-identity, and host foraging-guild on biodiversity of the microbiota. Microbe biodiversity was highly variable among conspecifics. Geographical location exhibited consistent effects, whereas host sex did not. Within each host guild, host species exhibited consistent differences in biodiversity of oral microbiota and of rectal microbiota. Oral microbe biodiversity was indistinguishable between guilds, whereas rectal microbe biodiversity was significantly greater in carnivores than in herbivores. The high intraspecific and spatial variation in microbe biodiversity necessitate a relatively large number of samples to statistically isolate the effects of environmental or host characteristics on the microbiota. Species-specific biodiversity of oral microbiota suggests these communities are structured by direct interactions with the host immune system via epithelial receptors. In contrast, the number of microbial taxa that a host gut supports may be driven by host diet-diversity or composition.

Introduction

Microbiota perform vital functions for their mammalian hosts, including nutrient acquisition, pathogen defense, and immune development [1–3]. Consequently, microbiota may be essential drivers of host evolution, affecting their physiology, immunocompetence, diet, and fitness [4]. Moreover, aspects of mammalian physiology, anatomy, behavior, diet, and niche affect which microbes encounter particular within-host habitats (e.g. skin, oral cavity, gastrointestinal tract). Consequently, these symbiotic associations likely represent coevolutionary relationships [1].

Understanding effects of environmental factors and host characteristics on the composition and biodiversity of microbiota may provide insights into the origin and maintenance of these symbiotic relationships. Host phylogeny, host diet, and environmental characteristics (especially those associated with the host as a habitat) are primary candidates to influence variation in the composition or biodiversity of microbiota [5–7]. Host phylogeny is a particularly attractive explanation, as it forms the basis for coevolutionary dynamics. Because organisms generally evolve via descent with modification, phylogenetic inertia gives rise to a priori expectations that more closely related species will be more similar to each other and that more distantly related species will be less similar to each other [8]. Consequently, host phylogeny may be an effective proxy for combinations of host characteristics that affect microbiota composition or biodiversity rather than an explanatory mechanism, per se.

Host diet has been a focal point for understanding the composition and biodiversity of gastrointestinal microbiota,

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which facilitate digestive processes and are exposed to ingested food. Consequently, intra- or inter-specific differences in host diet may result in differences in gastrointestinal microbiota due to exposure (i.e. animals with similar diets may consume similar microbiota) or due to the digestive functions provided by the microbiota [7, 9]. In addition, hosts that live in similar environments may be exposed to similar microbiota [9]. Important aspects of the environment of hosts may affect composition and biodiversity of their microbiota, including host abundance (resource abundance) and host community composition (resource diversity) as well as general environmental characteristics (e.g. roost type, habitat type, or abiotic factors).

Studies typically consider samples comprising intestinal contents, intestinal linings, or feces to represent the same microbial communities [2, 7, 10]. However, microbiota isolated from the mucosal layer of the intestines are distinct from those isolated from feces or intestinal contents [11]. Importantly, differences among microbiota from intestinal mucosa are closely associated with host evolutionary relationships, whereas differences among fecal microbiota are closely associated with dietary variation among hosts [11].

Oral microbiota provide benefits to the host, including prevention of infection by exogenous microorganisms, regulation of immune responses, and the conversion of dietary nitrates into nitrites that improve vascular health and stimulate gastric mucus production [12]. The oral environment (e.g. pH, immunoglobulins, lysozymes, temperature, nutrient sources, aerobic conditions) determines which microbes colonize and become minor or major components of the oral microbiota [13]. In addition, the microbiota can modify the environment, facilitating or preventing establishment by other microbes. Despite the importance of oral microbiota to hosts, they have rarely been studied in wild animals (but see [10]).

Bats as Hosts

Bats are an ideal host taxon for the study of variation in microbe biodiversity [2]. They represent the 2nd most species-rich order of mammals, are nearly cosmopolitan, are locally abundant, travel long distances between winter and summer ranges, and are functionally diverse [14]. Moreover, bats are important agents of pollination, seed dispersal, and pest control [15], and exhibit specializations to forage on nectar, fruit, insects, fish, small vertebrates, and blood [14]. However, functional traits and behaviors are evolutionarily conserved in bats, often confounding the ability to evaluate independent effects of diet or phylogeny on ecological patterns [16].

Understanding the composition and biodiversity of bat microbiota may be especially important because many bats live in proximity to humans [17], are reservoirs or vectors for many well-known zoonoses [18–20], and for some diseases their proximity to humans can affect infection rates in humans [21]. Bats use many human-dominated habitats: they feed on fruits in orchards, forage for insects around lights, and use buildings for maternity colonies, roosts, and hibernacula [17]. In addition, bats are highly vagile and capable of traveling long distances in a single night. This creates opportunities for exposure to novel microbes and dispersal of those microbes to new habitats or host communities [22]. In addition, microbiota may drive host evolution, physiology, and fitness [2]. For example, the successful evolution of new dietary strategies within a clade (e.g. the diversification of herbivorous strategies within the Phyllostomidae) may have been contingent on the functional diversity of their associated microbiota. Finally, the digestive physiologies of flying vertebrates (bats and birds) differ from those of non-volant vertebrates [23], including reliance on paracellular glucose absorption, resulting in mechanisms structuring their microbiota that differ from those of other terrestrial vertebrate groups.

Thirteen species of bat occur on Puerto Rico [24], including seven insectivores (*Eptesicus fuscus*, *Lasiurus borealis*, *Molossus molossus*, *Mormoops blainvillii*, *Pteronotus quadridens*, *P. parnellii*, *Tadarida brasiliensis*), a piscivore (*Noctilio leporinus*), a nectarivore (*Monophyllus redmani*), two frugivores (*Artibeus jamaicensis*, *Stenoderma rufum*), and two generalist herbivores (*Brachyphylla cavernarum*, *Erophylla sezekorni*). Bats that consume fruit, nectar, flowers, or pollen typically have diverse diets that differ in the identity of preferred dietary items, with *M. redmani* being primarily nectarivorous, *A. jamaicensis* and *S. rufum* being primarily frugivorous, and *E. sezekorni* and *B. cavernarum* being generalists, but with dietary composition changing seasonally because of associated variation in resource availability [24]. Puerto Rican insectivores belong to three families (Vespertilionidae, Molossidae, and Mormoopidae); the piscivore is a noctilionid; and phyllostomids are herbivorous. The Noctilionidae, Mormoopidae, and Phyllostomidae are members of the superfamily Noctilionoidea, whereas the Vespertilionidae and Molossidae are members of the superfamily Vespertilionoidea [25]. These systematic relationships decouple insectivory from phylogeny and may help disentangle the relative effects of evolutionary history and ecological function as drivers of microbiota composition and biodiversity. We grouped bats into broad foraging guilds (carnivores and herbivores) to evaluate effects of general diet on biodiversity of their microbiota.

Microbe Biodiversity

Few studies of microbiota from wild mammals have evaluated patterns of biodiversity among hosts, and most have used only richness or entropies (i.e. non-scaled, unitless diversity

measures). Analysis of entropies often provides misleading results and does not possess intuitive or desirable mathematical properties for statistical analysis [36]. Consequently, analyses based on entropies do not provide reliable results or interpretations [10, 27]. Transformation of entropies into Hill numbers facilitates effective interpretation, as all indices are in the same units (effective number of species) and have the necessary mathematical properties that facilitate logical comparison and statistical analysis [26, 28, 29]. The value of a Hill number is the number of species with equal abundance that is required to produce the empirical value of a metric [28]. As a result of transformation, all metrics are scaled from 1 to taxon richness, facilitating comparisons among metrics. Using Hill numbers and a suite of biodiversity metrics that differentially weight taxon abundances can determine if patterns are influenced by transient or incidental microbes, which affect richness but have little to no effect on abundance-weighted measures.

Studies with sufficient sample sizes from multiple locations, species, or foraging guilds to powerfully and simultaneously address multiple factors that affect composition or biodiversity of the microbiota in bats are rare (but see [10]) and typically focus on only gastrointestinal microbiota. To address these issues, we collected microbiota from oral and rectal samples from bats at three locations (hereafter called "sites") in Puerto Rico. We evaluated the relative importance of site, host sex, host species identity, and host foraging guild on biodiversity of oral and rectal microbiota separately. We used a hierarchical analytical design to evaluate these factors (Fig. S1, Online Resource 1). First, we evaluated effects of site (i.e. host population) and host sex on microbe biodiversity. Second, we evaluated the effect of host species identity on microbe biodiversity separately for bats within each of two broadly defined foraging guilds (carnivores versus herbivores). Finally, we evaluated the effect of host foraging guild on microbe biodiversity.

We expected dietary guild to have a larger impact on the biodiversity of rectal microbiota than on biodiversity of oral microbiota because sources of nutrients and energy (fats, carbohydrates, proteins) have a dominant effect on the composition and diversity of microbiota associated with the digestive tract [6]. In contrast, we expected biodiversity of only the oral microbiota to respond to host species identity and geographical site because oral microbiota are affected primarily by the interactions with the epithelia and exposure to local habitats (e.g. roost locations, animals that share a roost, hot cave versus cold cave).

Methods

Study Area and Sample Collection

Field work was conducted at three sites (Mata de Plátano, Río Encantado, and Aguas Buenas) in Puerto Rico (Fig. S2, Online Resource 1). Each is in an area characterized by limestone formations in which weathering has produced ridges, towers, fissures, sinkholes, and caves throughout the landscape. Although bats captured in a location may not be roosting in a single cave, all are using the same habitats and resources, meeting the criteria for a population. In addition, sites were a minimum of 20 km apart from each other (Fig. S2, Online Resource 1), greatly exceeding the typical home range size of Puerto Rican bats. For example, *A. jamaicensis* and *E. fuscus* typically have a home range covering < 30 square km and maintain some of the largest home ranges of Puerto Rican bats [24, 30]. Most species (e.g. *M. redmani*, *N. leporinus*, *S. rufum*, *P. quadridens*) maintain home ranges < 3 square km and exhibit fidelity to permanent roost structures [24, 31, 32].

The majority of sampling was conducted on the Mata de Plátano Nature Reserve in north-central Puerto Rico (18° 24.87' N, 66° 43.53' W). Mata de Plátano harbors two adjacent, well-studied caves (Culebrones and Larva). Culebrones is a structurally complex hot cave, with temperatures reaching 40 °C and relative humidity at 100%. It is home to about 300,000 bats representing six species [32]: three carnivores (*P. quadridens*, *P. parnellii*, *M. blainvillii*) and three herbivores (*M. redmani*, *E. sezekorni*, *B. cavernarum*). Bats were sampled at Culebrones for 28 nights from June to August 2017 using a harp trap placed adjacent to the cave opening. The harp trap was used at Culebrones because the cave has a single, small opening that funnels hundreds of thousands of bats through a small space as bats emerge during and after sunset.

Larva Cave is smaller, cooler, and less structurally complex than is Culebrones. Only 30−200 bats representing two species (*A. jamaicensis* and *E. fuscus*) roost there. Bats were sampled from Larva on seven occasions from June to August of 2017 using mist nets along the trail outside of the cave entrance and hand nets to capture bats inside the cave.

Río Encantado is home to Ramon Cave (18° 21.41' N, 66° 32.36' W), a large, cool cave known to support only *A. jamaicensis* [24]. The cave is 20 km southeast of Mata de Plátano. Bats were sampled at Río Encantado on six nights during July of 2017 using a harp trap near the cave entrance and mist nets along the trail leading to the cave.

Aguas Buenas is a cool cave located 70 km southeast of Mata de Plátano (18° 14.01' N, 66° 6.30' W). *Artibeus jamaicensis*, *B. cavernarum*, *M. redmani*, *P. quadridens*, *E. fuscus*, and *L. borealis* roost in or fly near the cave [24]. Bats were captured at Aguas Buenas on four nights in July and August of 2017 using mist nets at each of the two major flyways leading to the cave.

Species identity, sex, reproductive status, and mass were determined for each captured individual. Wing punches were collected for separate analyses, leaving a small hole in the wing of sampled individuals, thereby ensuring that each

microbial sample represented a unique individual. Cotton swabs were used to collect saliva from the mouth or feces from the rectum and anal region of each bat. Swabs were placed in individual cryovials and sent to the University of Connecticut at −80 °C in a dry ice shipper.

Microbiota Analysis

DNA was extracted using the DNeasy PowerSoil kit (Qiagen, Germantown, MD, USA). For every DNA kit, two negative reagent control samples were processed and sequenced. Swabs were shaved to maximize DNA output using sterile surgical blades, carbon steel, size 15 (Bard-Parker). The DNeasy PowerSoil QIAcube protocol was followed, and the vortexing step was substituted by a bead-beating step with the PowerLyzer 24 (45 s at 2000 RPM for one cycle) (Qiagen, Germantown, MD, USA).

The hypervariable V4 region of the 16S rRNA gene was amplified to characterize the microbiota [33]. The universal 16S primers 515F/806R were used to amplify the V4 region via PCR analysis [34]. For every 96-well plate PCR, a negative water control and three serial dilutions of positive gDNA mock community were included (Zymo Research, Irvine, CA, USA). The PCR reactions were setup in a Pre-PCR EpMotion 5073 (Eppendorf, Enfield, CT) with a HEPA filter. PCR was performed in triplicate, each reaction with a total volume of 25 µL. Each reaction contained 12.5 µL Phusion High-Fidelity PCR Master Mix with HF Buffer 2X concentration and 1 µL bovine serum albumin 20 mg/ml (New England BioLabs), 0.75 µL forward primer 10 µM, 0.75 µL reverse primer 10 µM, and up to 10 µL of DNA/molecular grade water in a final volume of 12.5 µL. A total of 10 ng DNA was added per reaction or up to 10 µL if the DNA concentration was too low. PCR was performed on a C1000 touch (BioRad, Hercules, CA) using the following parameters in a 384-well plate: denaturing step at 95 °C for three minutes, followed by 30 cycles of 95 °C for 45 s, 50 °C for 60 s, 72 °C for 90 s, and an extension step of 72 °C for 10 min. Triplicates were pooled using a post-PCR EpMotion 5073 with a HEPA filter. Subsequently, QIAxcel capillary electrophoresis (Qiagen, Germantown, MD, USA) was utilized to assess presence of PCR products and determine the V4 band concentration for library pooling. PCR samples with similar concentrations (< 5 ng/µL, 5–10 ng/µL, > 10 ng/µL) were pooled together. Library clean-up was performed using GeneRead Size Selection Kit (Qiagen, Germantown, MD, USA). Libraries were sequenced on an Illumina MiSeq at the UConn Microbial Analysis, Resources, and Services Facility. The reads were demultiplexed using the Illumina BaseSpace sequence hub and FASTQ files were downloaded for further data analysis.

Data were analyzed in R [35] using the dada2 package to process data, generate Amplicon Sequence Variants (ASV), and produce taxonomy tables. Forward and reverse reads were trimmed to 240 bp and 200 bp, respectively, and truncated using Q = 11 and no Ns were allowed. The taxonomy was assigned to each ASV using silva_nr_v128. The phangorn package [36] was used to generate phylogenetic trees from ASV tables. Further analyses and sample filtering were performed in phyloseq. In phyloseq, data were filtered using the package decontam [37] to remove bacterial sequences that were likely contaminants. Using the rarefy_even_depth function in phyloseq, microbiota count data were rarefied to sequencing depths of 5000, and 10,000 reads. Data were rarefied to these levels to optimize sampling completeness, while maximizing sample sizes for analyses. Increasing sequencing depth reduces the number of samples that meet the minimum requirements, resulting in reduced statistical power, but increases the relative completeness and number of rare ASVs included in samples. One small set of samples from a PCR plate where the EpMotion malfunctioned contained a reagent control that exceeded our rarefaction level and all of the associated samples were excluded from analysis. All of the other reagent and negative controls were well below the cutoff. Sequencing data of V4 region of the 16S rRNA gene is deposited in the NCBI Short Read Archive database under BioProject PRJNA602518 and accession numbers SRX7587313-7587772.

Quantitative Analysis

For each sample, we quantified biodiversity using four metrics based on ASVs: observed ASV richness, Shannon diversity, Camargo evenness, and Berger-Parker dominance [38]. These metrics represent a gradient from ignoring variation in abundance (richness) to weighting the number of taxonomic entities based on relative abundances (Shannon diversity, evenness) to using only the relative abundance of only the most dominant taxon (Berger-Parker dominance). Richness accounts for every microbial taxon in a sample and may be influenced by transient taxa ingested by the host or by non-adaptive microbes included in samples by happenstance. However, abundance-based metrics minimize the effect of transient or contaminant taxa on estimates of biodiversity, better reflecting the adaptive core microbiota. For ease of exposition, hereafter we refer to these metrics simply as "richness", "diversity", "evenness", and "dominance", and use "biodiversity" to refer to the general concept that comprises all four metrics. Each metric was expressed as its Hill number [26, 29]. Greater values for any Hill number represent greater biodiversity, including for dominance (i.e. larger values for Hill-transformed dominance indicate low dominance and greater biodiversity). Metrics that represent taxon counts (e.g. richness) are numbers equivalents and require no transformation [26]; however, abundance-weighted metrics require transformation. Numbers equivalents for

Shannon diversity, Camargo evenness, and Berger-Parker dominance were quantified following [26] with functions written in Matlab R2017a (ver. 9.2.0.556344).

We used a 2-way analysis of variance (ANOVA) with type II sums of squares to evaluate effects of site (i.e. host population) and host sex for each host species whose microbiota was effectively characterized for more than one population. Site and host sex were model I treatment factors. The microbiota for *A. jamaicensis* was characterized at all three caves; the microbiota for *E. sezekorni* was characterized at Mata de Plátano and Río Encantado; and the microbiota for *M. redmani* was characterized at Mata de Plátano and Aguas Buenas. For each host species without sufficient samples from multiple caves, but with samples for each sex, we used a general linear mixed-effects model (GLMM) to evaluate differences in microbe biodiversity between males and females with host sex as a fixed effect and site as a random factor to control for geographic variation and more powerfully evaluate differences in microbe biodiversity between sexes.

We used GLMMs to evaluate differences in microbe biodiversity among host species for each guild (i.e. only among carnivorous species and only among herbivorous species) and between host guilds. Host species or host guild was a fixed effect and site was modeled as a random factor to control for geographic variation and more powerfully evaluate species- or guild-level differences in microbe biodiversity. For each GLMM that identified a significant difference in microbe biodiversity between host species within a guild, we conducted a posteriori tests (Tukey's test with a Holm-Šidák adjustment) to identify consistent differences between each possible pair of host species. Because such a posteriori tests are less powerful than their associated GLMM and are protected in the sense that a posteriori tests were only executed when GLMMs were significant ($\alpha \leq 0.05$), we considered $P \leq 0.10$ as evidence for significant pairwise differences that contributed to overall observed differences in the associated GLMM.

For all analytical approaches, oral and rectal microbiota were evaluated separately for each sequencing depth (i.e. 5000 and 10,000 reads) and analyses were conducted separately for each metric of biodiversity. For analyses based on host foraging guild, all host species were included to best represent variation associated with all carnivorous or herbivorous hosts.

Results

Oral and rectal samples were collected from 331 bats, representing 10 species: three insectivorous mormoopids (*M. blainvillii*, *P. quadridens*, *P. parnellii*), one insectivorous vespertilionid (*E. fuscus*), one piscivorous noctilionid (*N. leporinus*), two frugivorous phyllostomids (*A. jamaicensis*, *S. rufum*), one nectarivorous phyllostomid (*M. redmani*), and two generalist herbivore phyllostomids (*B. cavernarum*, *E. sezekorni*). Samples were obtained from all 10 bat species at Mata de Plátano (155 individuals), nine species (all but *S. rufum*) at Río Encantado (101 individuals), and six species (75 individuals) at Aguas Buenas (*P. parnellii*, *N. leporinus*, *A. jamaicensis*, *M. redmani*, *B. cavernarum*, and *E. sezekorni*). We obtained sequence depths ≥ 5000 reads from less than half of those samples. Specifically, 107 and 90 oral samples yielded sequencing depths of at least 5000 and 10,000 reads, respectively; and 121 and 105 rectal samples yielded sequencing depths of at least 5000 and 10,000 reads, respectively.

Oral microbiota comprised 2257 and 1944 ASVs in samples with sequencing depths of 5000 and 10,000 reads, respectively. Rectal microbiota comprised 4032 and 4023 ASVs in samples with sequencing depths of 5000 and 10,000 reads, respectively. The reduction in cumulative number of ASVs between sequencing depths of 5000 and 10,000 is due to the smaller number of samples available for analysis. Bacteria represented over 98.8% of the ASVs in oral and rectal microbiota from each host species, with Archaea comprising the remainder of the microbiota.

Streptococcus and *Staphylococcus* were among the most abundant and frequent genera in oral microbiota from five (*A. jamaicensis*, *B. cavernarum*, *E. sezekorni*, *P. quadridens*, and *S. rufum*) and three (*B. cavernarum*, *E. fuscus*, and *E. sezekorni*) host species, respectively. No other genus was frequent and abundant in the oral microbiota of more than two host species.

Lysinibacillus (*B. cavernarum*, *E. fuscus*, *E. sezekorni*, *M. redmani*, and *P. portoricensis*), *Mycoplasma* (*A. jamaicensis*, *E. sezekorni*, *P. portoricensis*, and *S. rufum*), *Helicobacter* (*B. cavernarum*, *E. fuscus*, *E. sezekorni*, and *M. redmani*), and *Staphylococcus* (*B. cavernarum*, *E. fuscus*, and *P. portoricensis*) were the only genera that were abundant and frequent in the rectal microbiota of more than two host species. Complete lists of the named genera identified from the oral or rectal microbiota from each host species are in supplementary materials (Table S1, Online Resource 2).

Biodiversity was highly variable among individuals within each host species. Using a sequencing depth of 5000 as an example, maximum richness from an individual host for oral microbiota was 3 to 33 (mean of 10) times greater than the minimum richness within host species. Similarly, maximum richness of rectal microbiota from an individual host was 11 to 85 (mean of 28) times greater than the minimum within host species. Similar variation was observed within each host species for oral and rectal microbiota based on diversity, evenness, and dominance metrics. Despite this variation seeming extreme, it is important to remember what it indicates: that the microbiota of some host individuals are

dominated numerically by a few ASVs, whereas the microbiota of other host individuals are diverse with no numerically dominant ASV.

In general, rectal microbiota were more diverse than their associated oral microbiota for the same host species, with the insectivorous *P. parnellii* being a notable exception (Table 1). In general, biodiversity of the more biodiverse microbiota (oral or rectal) was less than twice as great as its companion microbiota; however, *E. fuscus* (an insectivore) harbored rectal microbiota that were more than four times as biodiverse as its oral counterpart.

Insectivores had both the least (*E. fuscus*) and greatest (*P. quadridens*, *P. parnellii*) oral microbiota biodiversity. In contrast, frugivores (*A. jamaicensis*, *E. sezekorni*)

Table 1 Mean biodiversity of oral and rectal microbiomes for each of 10 bat species in Puerto Rico as well as for all bats in each of two foraging guilds (carnivores and herbivores) regardless of species

Foraging guild Family	Richness		Shannon diversity		Carmargo evenness		B–P dominance	
Species (oral, rectal sample sizes)	Oral	Rectal	Oral	Rectal	Oral	Rectal	Oral	Rectal
5000 reads								
Carnivores	**61.62**	**122.93**	**11.39**	**24.98**	**12.25**	**25.63**	**2.67**	**4.60**
Mormoopidae								
Mormoops blainvillii (0, 2)	–	22.50	–	1.94	–	1.90	–	1.16
Pteronotus parnellii (5, 9)	200.40	170.00	40.05	29.45	44.64	32.72	4.60	4.72
Pteronotus quadridens (2, 1)	180.93	329.00	34.64	149.16	40.40	123.14	5.08	25.51
Noctilionidae								
Noctilio leporinus (11, 8)	33.00	96.25	5.91	18.96	4.79	18.24	2.42	4.83
Vespertilionidae								
Eptesicus fuscus (11, 9)	16.09	99.00	2.77	17.19	2.31	19.55	1.48	2.70
Herbivores	**43.59**	**49.03**	**9.68**	**7.03**	**8.44**	**7.62**	**2.94**	**2.11**
Phyllostomidae								
Artibeus jamaicensis (45, 53)	32.82	38.98	8.20	6.13	6.67	7.13	2.93	1.62
Brachyphylla cavernarum (15, 13)	46.53	77.38	8.50	9.31	6.87	10.33	3.11	2.59
Erophylla sezekorni (8, 24)	57.75	48.71	5.28	9.38	6.16	10.53	1.81	2.28
Monophyllus redmani (9, 1)	82.89	86.00	23.67	24.37	22.69	23.84	3.46	4.66
Stenoderma rufum (1, 1)	35.00	185.00	4.63	24.41	3.69	30.04	2.94	4.01
10,000 reads								
Carnivores	**66.62**	**153.24**	**11.04**	**22.02**	**12.20**	**25.12**	**2.64**	**3.70**
Mormoopidae								
Mormoops blainvillii (0, 1)	–	28.00	–	2.59	–	2.34	–	1.28
Pteronotus parnellii (1, 7)	231.75	225.43	45.76	30.80	50.68	36.56	6.42	4.47
Pteronotus quadridens (4, 0)	208.00	–	10.79	–	38.08	–	1.56	–
Noctilionidae								
Noctilio leporinus (11, 6)	36.27	113.17	5.86	15.45	4.76	16.18	2.39	4.09
Vespertilionidae								
Eptesicus fuscus (10, 7)	19.80	133.29	2.88	21.63	2.41	24.60	1.51	2.95
Herbivores	**42.29**	**57.35**	**7.43**	**7.14**	**6.35**	**7.89**	**2.71**	**2.11**
Phyllostomidae								
Artibeus jamaicensis (39, 49)	33.72	46.92	7.69	4.95	6.30	5.18	2.79	1.86
Brachyphylla cavernarum (14, 11)	54.21	99.91	8.57	10.43	6.94	11.77	3.06	2.80
Erophylla sezekorni (7, 23)	66.86	53.43	5.24	9.56	6.60	10.89	1.75	2.25
Monophyllus redmani (3, 0)	45.33	–	2.00	–	2.25	–	1.43	–
Stenoderma rufum (1)	36.00	200.00	4.62	23.55	3.66	29.52	2.01	4.07

Biodiversity was quantified using each of four metrics based on Amplified Sequence Variants (richness, Shannon diversity, Camargo evennes, Berger-Parker dominance) and expressed as Hill numbers. Guild-level values are bold

had the rectal microbiota with the least biodiversity, and insectivores (*P. quadridens*, *P. parnellii*, *E. fuscus*) had the rectal microbiota with the greatest biodiversity (Table 1).

Host sex did not exhibit a significant effect on richness of oral or rectal microbiota in any of 19 analyses (Table 2). In contrast, host sex had a consistent effect on diversity, evenness, and dominance of the oral microbiota of *B. cavernarum* and exhibited significant effects on diversity and dominance of rectal microbiota of *A. jamaicensis* (Table 2). Consistent effects of site on oral microbiota only manifested for *A. jamaicensis*. At least one metric of rectal microbe biodiversity responded to site for *A. jamaicensis* (richness) and *E. sezekorni* (richness and evenness; Table 2). Oral and rectal microbe biodiversity was generally greater from *A. jamaicensis* at Río Encantado than from *A. jamaicensis* at Mata de Plátano or Aguas Buenas (Fig. 1). For *E. sezekorni*, rectal microbe biodiversity was greater at Río Encantado than at Mata de Plátano. Microbe biodiversity from *M. redmani* did not differ between sites (Table 2).

Within each host guild, host species differed in oral microbe biodiversity at each sequencing depth; however, interspecific host differences in rectal microbe biodiversity decreased among carnivores with increasing sequence depth (Table 3). Based on post hoc analyses, we have stronger evidence for consistent species-specific differences in oral microbe biodiversity within each guild than for species-specific differences in rectal microbe biodiversity within each guild (Table S2, Online Resource 1). No evidence suggests that guild-specific differences exist in oral microbe biodiversity, whereas rectal microbe biodiversity differed significantly between guilds (Table 3). Rectal microbe biodiversity in carnivores was about thrice that in herbivores (Table 1; Fig. 2).

Discussion

Considerable intraspecific variation characterized microbe biodiversity, even after controlling for geography or sex of the host individual. These results mirror those for fecal and gastrointestinal microbiota from vespertilionid bats of Slovenia [27] and from emballonurid, molossid, mormoopid, phyllostomid, and vespertilionid bats from Costa Rica [11], for which variation among conspecific hosts was high. This suggests that studies relying on a few samples per host species [7, 9] do not accurately capture variation in biodiversity or composition of microbiota within populations. Consequently, ecological conclusions based on such small samples may not be reliable because estimates of biodiversity (and likely composition) likely are not accurate, and statistical power to detect differences in any metric would be low. Importantly, stochastic variation associated with transient or non-adaptive microbes in samples could affect measures of richness, but would have negligible effects on abundance-based measures of biodiversity. Patterns of biodiversity in the oral and rectal microbiota of Puerto Rican bats were not appreciably different based on richness versus abundance-based metrics (Tables 2 and 3). Moreover, abundance-based biodiversity exhibited more consistent responses to host sex and to geography than did richness, suggesting that detected patterns represent responses of adaptive members of the microbiota rather than effects of non-adaptive transient microbes present in samples.

Greater microbe biodiversity in a host species could arise from: (1) an increase in the number of phyla or classes of microbes, or (2) an increase in the number of ASVs that belong to the same phyla or classes of microbes (i.e. not an increase in higher-level taxonomic biodiversity). For both oral and rectal microbiotas, the latter scenario occurred. Host species with greater microbe biodiversity (e.g. *P. parnellii*, *P. quadridens*, *M. redmani*) typically harbored more ASVs belonging to the same phyla as those present in hosts with low microbe biodiversity. Richness of Archaea and Bacteria at the host-species level (i.e. data combined for all hosts belonging to the same species) were highly correlated (oral, $R = 0.928$, $P < 0.001$; rectal, $R = 0.690$; $P = 0.027$). Similarly, pairwise correlations between richness of different phyla at the host-species level indicate that positive associations predominate (i.e. an increase in microbe richness is associated with an increase in richness for most of the phyla). Seventy and 56% of pairwise correlations of relative abundances of phyla were strongly positive ($R > 0.50$) in oral and rectal microbiota, respectively. Such correlations also characterize vespertilionid, rhinolophid, and miniopterid bats from Slovenia [27].

Bats are reservoirs or vectors for many well-known zoonoses [18–21], and we detected many genera that include species that are pathogens to humans or to other animals, including *Aeromonas*, *Bacillus*, *Clostridium*, *Escherichia*, *Flavobacterium*, *Fusobacterium*, *Haemophilus*, *Klebsiella*, *Moraxcella*, *Mycobacterium*, *Mycoplasma*, *Neisseria*, *Pseudomonas*, *Staphylococcus,*and *Streptococcus* (Table S1, Online Resource 2). However, this information is not sufficient to determine if these bats carry pathogens and serve as vectors for zoonotic disease. The V4 region of the 16S rRNA gene used in this study is widely used in studies that determine the composition of the microbiota in animals, plants, soil, and other habitats. While these short regions allow thousands of sequences to be obtained for each sample, they contain limited taxonomic information and typically do not provide species level resolution. Thus, alternative approaches that sequence entire rRNA genes or the metagenome are needed to confidently determine that these bats carry potential human pathogens.

Table 2 Results (*P*-values) of 1-way generalized linear mixed-effects models (for analyses of host sex only with site as a model II treatment factor) or 2-way analyses of variance with type II sums of squares (for analyses of site and host sex) evaluating the effects of site or host sex on microbiota biodiversity

Host species	Oral microbiomes			Rectal microbiomes		
Sequence depth						
Biodiversity index	Site	Sex	Site × sex	Site	Sex	Site × sex
Pteronotus parnellii						
5000						
Richness	–	0.561	–	–	0.852	–
Shannon diversity	–	0.714	–	–	0.815	–
Camargo evenness	–	0.709	–	–	0.668	–
B-P dominance	–	0.586	–	–	0.953	–
10,000						
Richness	–	–	–	–	0.599	–
Shannon diversity	–	–	–	–	0.897	–
Camargo evenness	–	–	–	–	0.873	–
B-P dominance	–	–	–	–	0.867	–
Noctilio leporinus						
5000						
Richness	–	0.709	–	–	0.519	–
Shannon diversity	–	0.713	–	–	0.945	–
Camargo evenness	–	0.800	–	–	0.945	–
B-P dominance	–	0.487	–	–	0.902	–
10,000						
Richness	–	0.726	–	–	–	–
Shannon diversity	–	0.746	–	–	–	–
Camargo evenness	–	0.926	–	–	–	–
B-P dominance	–	0.316	–	–	–	–
Artibeus jamacensis						
5000						
Richness	0.186	0.406	0.121	**0.009**	0.788	0.999
Shannon diversity	**0.005**	0.891	0.141	0.319	**0.036**	0.817
Camargo evenness	**0.018**	0.792	0.119	0.141	0.140	0.712
B-P dominance	**0.002**	0.914	0.543	0.845	**0.007**	0.624
10,000						
Richness	0.383	0.217	0.217	**0.003**	0.741	0.951
Shannon diversity	**0.009**	0.379	0.277	0.230	**0.034**	0.735
Camargo evenness	**0.045**	0.356	0.242	0.098	0.139	0.695
B-P dominance	**0.003**	0.515	0.904	0.725	**0.007**	0.458
Brachyphylla cavernarum						
5000						
Richness	–	0.789	–	–	0.650	–
Shannon diversity	–	**0.027**	–	–	0.906	–
Camargo evenness	–	**0.045**	–	–	0.617	–
B-P dominance	–	0.064	–	–	0.321	–
10,000						
Richness	–	0.314	–	–	0.539	–
Shannon diversity	–	**0.036**	–	–	0.791	–
Camargo evenness	–	0.065	–	–	0.546	–
B-P dominance	–	**0.032**	–	–	0.292	–
Erophylla sezekorni						
5000						
Richness	–	0.392	–	0.078	0.194	0.093
Shannon diversity	–	0.555	–	0.103	0.273	0.207

Table 2 (continued)

Host species						
Sequence depth	Oral microbiomes			Rectal microbiomes		
Biodiversity index	Site	Sex	Site × sex	Site	Sex	Site × sex
Camargo evenness	–	0.345	–	0.057	0.225	0.124
B-P dominance	–	0.821	–	0.071	0.130	0.286
10,000						
Richness	–	0.338	–	**0.030**	0.102	**0.025**
Shannon diversity	–	0.696	–	0.065	0.211	0.120
Camargo evenness	–	0.325	–	**0.028**	0.152	0.055
B-P dominance	–	0.495	–	0.061	0.122	0.222
Monophyllus redmani						
5000						
Richness	0.113	0.969	0.841	–	–	–
Shannon diversity	0.164	0.707	0.735	–	–	–
Camargo evenness	0.160	0.824	0.829	–	–	–
B-P dominance	0.193	0.570	0.689	–	–	–

Analyses were conducted separately for each combination biodiversity metric, sample type (oral or rectal), and sequencing depth. Significant results ($P \leq 0.05$) are bold

Effects of Host Sex

Host sex has the potential to affect microbe biodiversity because of sex-specific differences in social organization and diet. Harems, comprising several adult females with one adult male, are common social structures for noctilionid and phyllostomid bats, whereas maternity colonies, comprising adult females and their offspring, are common in mormoopid and vespertilionid bats [39, 40]. Most adult males are solitary in both of these social systems. The diets of male and female bats can differ seasonally, especially during periods of pregnancy and lactation when females target food sources that are higher in energy and protein [41]. Despite sampling during the reproductive season, when these sex-based ecological differences manifest most strongly, we found little evidence of differences in microbe biodiversity between sexes (Table 2). When evidence of differences in microbe biodiversity did manifest (i.e. oral microbiota of *B. cavernarum* and rectal microbiota of *A. jamaicensis*), those differences reflected the relative abundances of ASVs (diversity, evenness, or dominance) and not the number of ASVs (richness). Similarly, the microbiota from 12 species of Slovenian vespertilionid exhibited no differences between the sexes [27].

Effects of Geographical Location

Despite the potential for environmental factors (e.g. roost environment, abundance and diversity of hosts in the roost) to affect oral microbe biodiversity, only *A. jamaicensis* exhibited site-specific differences (Table 2; Fig. 1). These differences may be related to population size or to host species diversity in associated roosts. Oral microbiota from *A. jamaicensis* in Río Encantado had the greatest biodiversity, whereas those from Mata de Plátano (Larva Cave) had the lowest. The population of *A. jamaicensis* at Río Encantado was greater than at other locations, especially compared to that at Mata de Plátano. However, population sizes differed between sites for other host species without realizing significant differences in oral microbe biodiversity. This suggests that host abundance or diversity are not the major factors determining oral microbe biodiversity in bat populations.

Rectal microbiota of each host species exhibited site-specific variation in biodiversity (Table 2; Fig. 1). In *A. jamaicensis*, rectal microbiota exhibited patterns similar to those observed for oral microbiota, with greater biodiversity associated with larger populations from roosts with greater bat species richness. In contrast, rectal microbiota from *E. sezekorni* exhibited greater biodiversity from Río Encantado than from Mata de Plátano, with the former harboring fewer individuals than the latter. Even though host abundance or biodiversity may not directly affect microbe biodiversity, they may serve as proxies for important ecological factors. For example, bat abundance or diversity are often related to the diversity or abundance of dietary items or habitat types used by resident bats [42], and diet or habitat diversity may influence spatial patterns of biodiversity in microbiota. Alternatively, microbe biodiversity within sites may represent legacies of historical factors, such as the effects of hurricane-induced disturbances on bat populations and communities [32]. The microbiota of tropical African bats exhibited similar patterns, with ecological factors explaining variation in the composition of oral, gut, and skin microbiota [10].

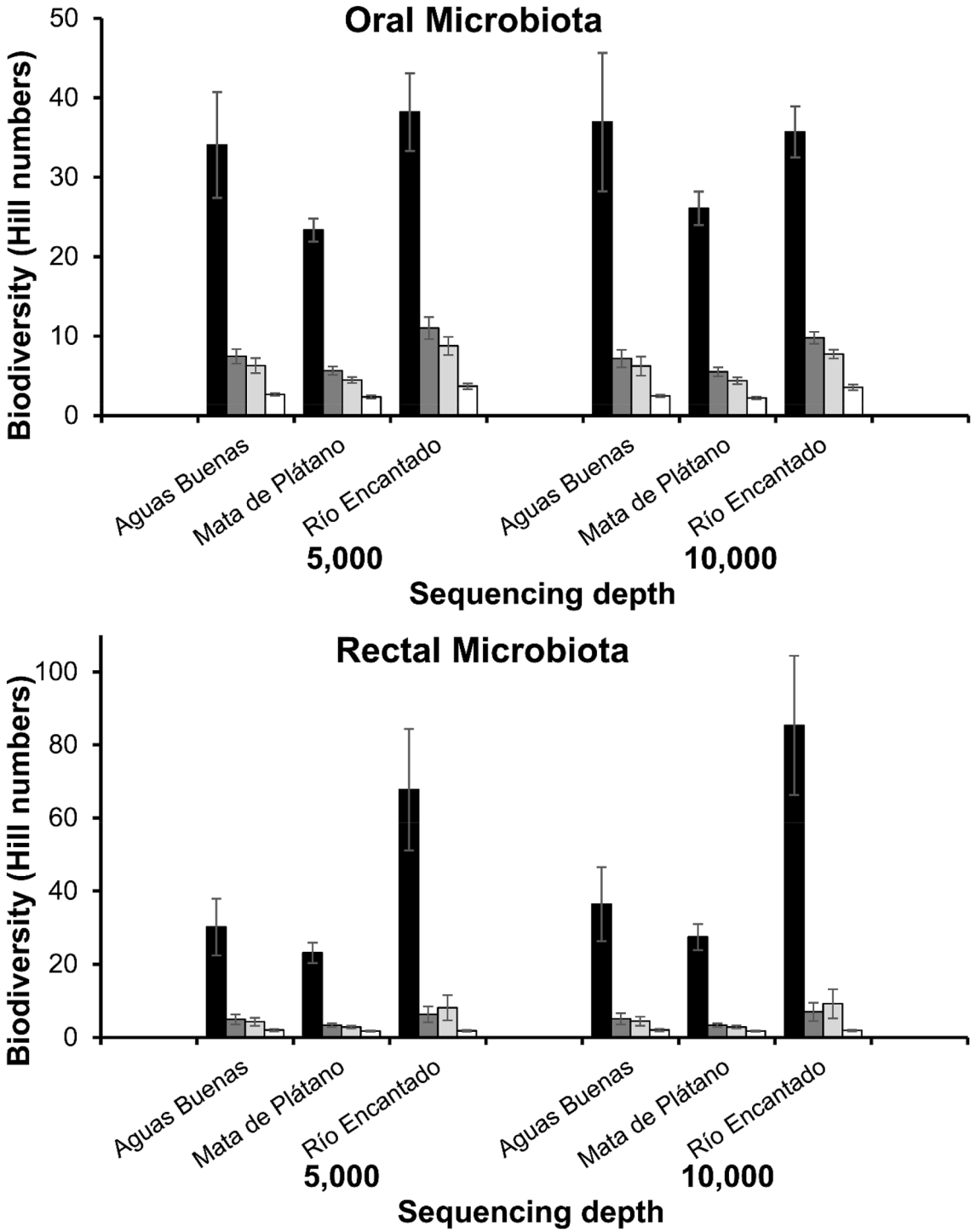

Fig. 1 Aspects of biodiversity (ASV richness, black bars; Shannon diversity, dark gray bars; Camargo evenness, light gray bars; Berger-Parker dominance, white bars) expressed as Hill numbers for oral and rectal microbiota from *Artibeus jamaicensis* at Aguas Buenas, Mata de Plátano, and Río Encantado at sequencing depths of 5000 or 10,000 reads. Error bars are ± 1 SE. In general, metrics of biodiversity for oral microbiota were least at Mata de Plátano. For rectal microbiota, only richness differed among sites, with Río Encantado exhibiting the greatest biodiversity

Table 3 Results (*P*-values) of general linear mixed-effects models evaluating the effect of host species or host guild on microbiota biodiversity

Sequencing depth Biodiversity index	Comparison of species within guilds				Comparison between guilds	
	Oral microbiome		Rectal microbiome			
	Carnivores	Herbivores	Carnivores	Herbivores	Oral microbiome	Rectal microbiome
5000						
Species richness	**< 0.001**	**0.013**	0.143	**0.010**	0.203	**< 0.001**
Shannon diversity	**< 0.001**	**0.007**	**< 0.001**	0.060	0.702	**< 0.001**
Camargo evenness	**< 0.001**	**0.004**	**0.009**	0.071	0.359	**< 0.001**
B-P dominance	**< 0.001**	0.134	**< 0.001**	**0.017**	0.412	**< 0.001**
10,000						
Species richness	**< 0.001**	**0.045**	0.363	**0.004**	0.115	**< 0.001**
Shannon diversity	**< 0.001**	**0.012**	0.589	0.085	0.799	**< 0.001**
Camargo evenness	**< 0.001**	0.258	0.534	0.070	0.265	**< 0.001**
B-P dominance	**< 0.001**	**0.009**	0.575	**0.029**	0.095	**< 0.001**

Effect of host species was evaluated separately for each guild

Species and guild were model 1 treatment factors (i.e. fixed effects) and cave was a model II treatment factor (i.e. random effects). Analyses were conducted separately for each combination of biodiversity metric, sample type (oral or rectal), and sequencing depth. Significant results ($P \leq 0.05$) are bold

Although confident identification of causal mechanisms that drive spatial variation in microbe biodiversity is challenging, results from multiple studies [10, 11, 27] indicate that spatial environmental variation must be considered when evaluating aspects of biodiversity of host microbiota.

Effects of Host Species or Guild on Biodiversity of Oral Microbiota

Within each host guild, species-specific differences characterized biodiversity of oral microbiota. In contrast, guild-specific differences did not characterize oral microbiota (Table 3). This combination of results indicates that oral microbe biodiversity is unrelated to host diet for Puerto Rican bats. For carnivores, nearly all pairwise comparisons of oral microbe biodiversity between host species were significant (Table S2, Online Resource 1), whereas, pairwise differences in oral microbe biodiversity between herbivorous bat species were related to differences between *M. redmani* (a nectarivore with high oral microbe biodiversity) and other herbivores.

Patterns of oral microbe biodiversity may be structured by processes similar to those that structure the microbiota from other mucosal surfaces (e.g. nose, mouth, lungs, gastrointestinal tract). The microbiota of the mucosal lining of the intestines directly interacts with the host immune system through receptors in the intestinal epithelia [43]. Direct sampling of the intestinal mucosa showed a strong relationship between composition of the microbiota and host phylogeny in Belizean bats [11]. The species-specific biodiversity observed for oral microbiotas within each guild of bats in Puerto Rico likely represents a similar coevolutionary association between hosts and their microbes. The five carnivorous species represent three families (Mormoopidae, Vespertilionidae, and Noctilionidae), which likely contribute to the preponderance of significant pairwise differences in the biodiversity of oral microbiota. In contrast, the lower frequency of pairwise differences in oral microbe biodiversity among herbivorous species may arise because they represent a single family (Phyllostomidae).

Effects of Host Species or Guild on Biodiversity of Rectal Microbiota

Species-specific differences of rectal microbiota within host guilds exhibited two patterns: (1) species-specific differences were more consistent at lower sequencing depths than at greater sequencing depths and (2) species-specific differences were observed more consistently between species of herbivore than between species of carnivore (Table 3). In contrast, consistent differences in biodiversity occurred between the rectal microbiota of carnivores and herbivores (Table 3). In concert, these results suggest that the biodiversity of rectal microbiota is molded to host diet. Regardless of metric, the biodiversity of rectal microbiota of carnivores was nearly twice as great as that from herbivores (Table 1; Fig. 2). Importantly, the lack of differences in biodiversity among species within host foraging guilds does not suggest that the composition of rectal microbiota is the same for these host species. Indeed, microbe composition may differ among host species within a guild, with different microbial taxa performing the same function in different host species. However, the number of microbial taxa that a host supports may be contingent on the general diet of the host species

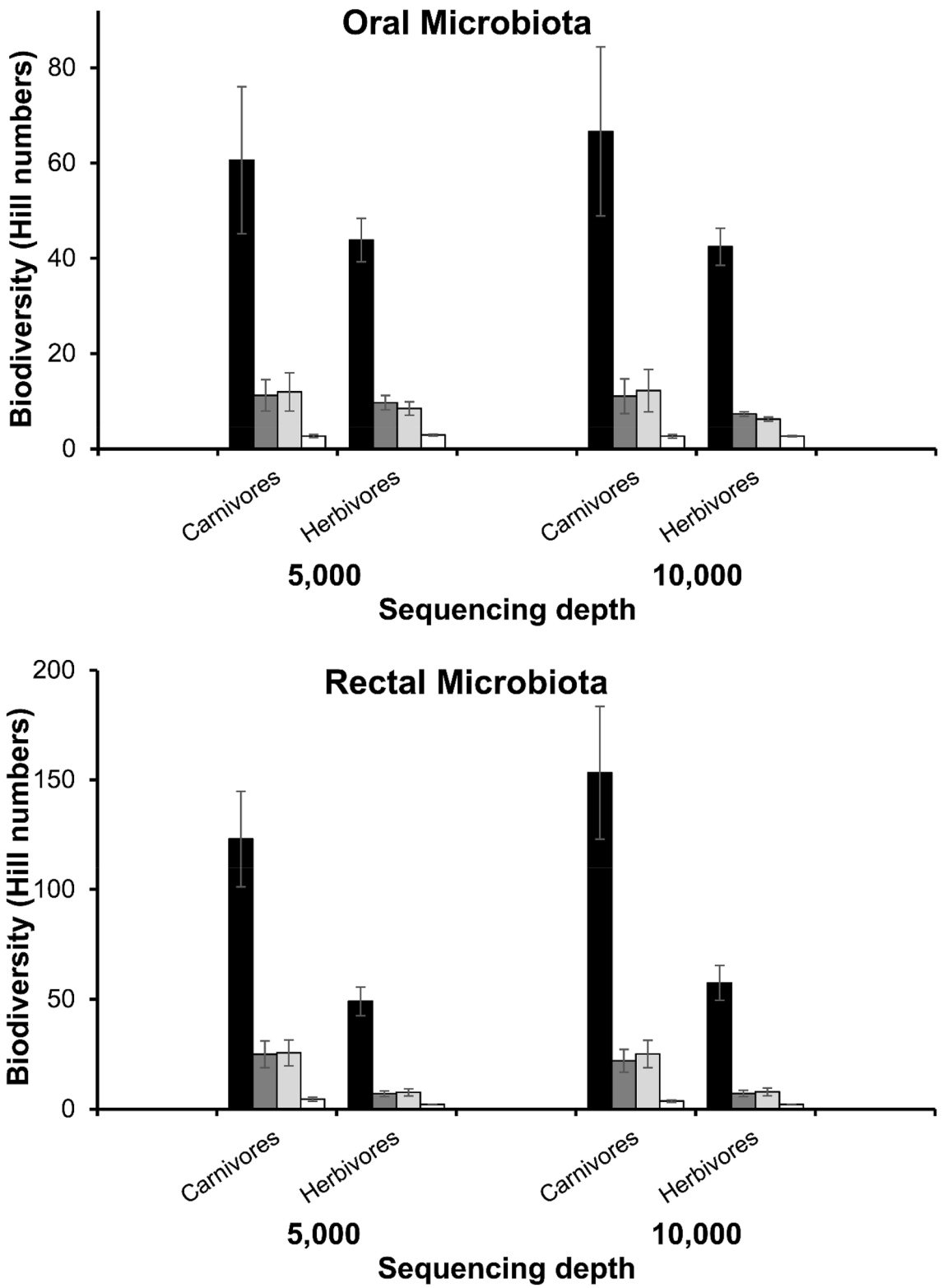

Fig. 2 Aspects of biodiversity (ASV richness, black bars; Shannon diversity, dark gray bars; Camargo evenness, light gray bars; Berger-Parker dominance, white bars) expressed as Hill numbers for oral and rectal microbiota from carnivorous and herbivorous bats at sequencing depths of 5000 or 10,000 reads. Error bars are ± 1 SE. In general, metrics of biodiversity did not differ between foraging guilds for oral microbiota, whereas metrics of biodiversity were significantly greater in carnivores than in herbivores for rectal microbiota

(i.e. the number and kinds of functions a host requires of its microbiota). This is consistent with the assembly of soil and plant microbiota, in which metacommunities contained fixed fractions of coexisting families that were determined by the available carbon sources [44]. Despite consistent familial level structure, these assembled microbiota exhibited exceptional variation in taxonomic composition with the same functions performed in each microbiota, but done so by different taxa within each family.

Microbiota associated with the digestive system from insectivorous bats are more biodiverse than those from their herbivorous counterparts in Guatemala [7], Mexico [9] and Puerto Rico (Tables 1 and 3). Greater microbe biodiversity in carnivorous bats contrasts with theory based on the study of a wide array of mammals (e.g. ruminants, primates, carnivores). Three general predictions have been postulated [45]: (1) herbivores should have the most complex gut morphologies and most diverse microbiota; (2) carnivores should have the most simple gut morphologies and least diverse microbiota; and (3) omnivores should have intermediate levels of gut complexity and microbe biodiversity. As an adaptation for flight, all bats have shorter intestines and shorter food-retention times than do similarly sized non-volant mammals [23, 46]. Nonetheless, herbivorous bats still have slightly longer intestines than do their carnivorous counterparts of similar size [47]. Herbivorous bats generally consume nectar and fruits that primarily contain simple sugars and carbohydrates, resulting in brief retention times (i.e. < 60 min [48, 49]). Moreover, herbivorous bats rely on paracellular absorption for > 70% of glucose absorption, which may explain why these bats have relatively depauperate rectal microbiota [23, 50]. In contrast, the high protein, lipid, and nutrient content of insectivorous diets may result in high microbe biodiversity due to the variety of carbon and energy sources available [9].

Conclusions

High-variation in microbe biodiversity among conspecific hosts suggests that individual-level host traits and behaviors affect associated microbiota, reflecting the individual histories of exposure via difference in roosting habitat, social interactions, and diet. That these patterns are consistent for all metrics of biodiversity suggests that this variation likely reflects the adaptive microbiota and is not caused by rare or transient, non-adaptive microbes. Although descriptive studies provide insights based on a few samples from each host species, research designed to explore the ecological dynamics of microbiota should account for high intraspecific variation by increasing the number of samples collected from host populations. Despite effects of host ecology and evolutionary history on their microbiota, microbe composition and biodiversity are also affected by spatial phenomena, primarily via host-environment interactions.

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Author Contributions MRW and JG conceived the project, and with SJP and ARS crafted the experimental design. ARS conducted sample collection. AFH conducted laboratory analyses. SJP conducted statistical analyses and wrote the manuscript. All authors critically reviewed multiple drafts of the manuscript.

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Data Availability Sequencing data of V4 region of the 16S rRNA gene has been deposited in the NCBI Short Read Archive database under BioProject PRJNA602518 and accession numbers SRX7587313-7587772.

Code Availability Not applicable.

Declarations

Conflict of Interest The authors have no relevant financial or non-financial interests to disclose.

Ethical Approval All methods were approved by the University of Connecticut Institutional Animal Care and Use Committee (IACUC, protocol A15-032).

Consent to Participate Not applicable.

Consent for Publication Not applicable.

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