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Gut Microbial Diversity and Ecological Specialization in Four Sympatric Lemur Species Under Lean Conditions

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Abstract

The gut microbiome is gaining recognition for its role in primate nutrition, but we stand to benefit from microbiome comparisons across diverse hosts and environmental conditions. We compared gut microbiome structure in four lemur species from four phylogenetic lineages, including 9 individual mouse lemurs (*Microcebus danfossi*), 6 brown lemurs (*Eulemur fulvus*), 20 sifakas (*Propithecus coquereli*), and a single sportive lemur (*Lepilemur grewcockorum*). In northwestern Madagascar, these species are sympatric, but use different feeding strategies to cope with environmental challenges, including relying on tree gums and insects (mouse lemurs), and some vs. significant leaf matter (brown lemurs vs. sifakas and sportive lemurs). From one fecal sample collected per lemur in the dry season in the Anjajavy Forest, we determined gut microbiome diversity, variability, and membership via 16S rRNA sequencing. The lemurs harbored strongly species-specific gut microbiomes. Brown lemurs showed more diverse and generalized consortia; mouse lemurs, sifakas, and the sportive lemur had less diverse consortia with more distinct memberships. Consistent with their fallback foods, mouse lemur microbiomes included taxa putatively associated with gum and insect digestion, whereas those of sifakas and the sportive lemur showed stronger and distinct signatures of leaf fiber and secondary compound metabolism. These results point to feeding strategy, intertwined with host phylogeny, as a driver of

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gut microbiome composition, but highlight real-time dietary specificity as a contributing driver of microbiome diversity. While illuminating how gut microbiomes facilitate host nutrition on challenging foods, these results help explain how ecologically diverse primates living in sympatry may differentially cope with seasonal or stochastic lean times.

Keywords *Eulemur* · Feeding ecology · *Lepilemur* · Microbiome · *Microcebus* · *Propithecus*

Introduction

The primate gut microbiome performs metabolic functions that can help hosts meet their nutritional and energetic demands (Clayton *et al.,* 2018). For example, microbes along the gastrointestinal tract can variably ferment recalcitrant dietary fibers and produce energetic short-chain fatty acids, metabolize plant secondary compounds and xenobiotics, synthesize vitamins and amino acids, and contribute to nitrogen cycling (Dearing & Kohl, 2017; Flint *et al.,* 2012; LeBlanc *et al.,* 2013; Oliphant & Allen-Vercoe, 2019; Wong *et al.,* 2006). The gut microbiomes of herbivores and folivores have gained much attention for their crucial role in processing plant fiber (e.g., Amato *et al.,* 2019; Dearing & Kohl, 2017); yet primates characterized by other ecological strategies may likewise rely heavily on their gut microbes for nutritional success and survival. Indeed, microbial and metagenomic plasticity may provide crucial mechanisms by which vertebrates can cope with environmental change (Alberdi *et al.,* 2016), including dietary diversity and hypervariable food availability across seasons or stochastic events. To better understand how gut microbes may help diverse primates cope with environmental challenge, we compare gut microbiome structure in four sympatric lemur species with different feeding strategies during the harsh dry season in northwest Madagascar.

Across species, there is accruing evidence that both feeding strategy and real-time diet shape microbiome features, although they do so at different scales (Greene, Bornbusch, *et al.,* 2019a; Rojas *et al.,* 2021). Whereas feeding strategies are characteristic of species and reflect aspects of gut morphology and dietary repertoires, many primates forage flexibly depending on current resource availability (Lambert & Rothman, 2015; Nowak & Lee, 2013). Therefore, concurrent diets may reflect variable subsets of foodstuffs associated with, or even outside of, a given feeding strategy. In general, the links between feeding strategies and gut microbiomes are intertwined over evolutionary time (Ley *et al.,* 2008; Nishida & Ochman, 2018; Rojas *et al.,* 2021). Primate species and lineages thus harbor distinct microbial assemblages that differently reflect host digestive physiology and dietary repertoires (Amato *et al.,* 2019; Greene, Bornbusch, *et al.,* 2019a; Perofsky *et al.,* 2019).

Within the constraints imposed by host feeding strategy and phylogeny, the diet items consumed by hosts further shapes the primate gut microbiome within species. For example, gut communities track the seasonal availability or consumption of foodstuffs, including leaves, grasses, fruits, and insects (Amato *et al.,* 2015; Baniel *et al.,* 2021; Gomez *et al.,* 2016; Hicks *et al.,* 2018; Orkin *et al.,* 2019; Springer *et al.,* 2017). Direct or indirect proxies for regional dietary intakes, such as habitat location, type, and

quality, can likewise predict primate microbiome diversity and composition (Barelli *et al.,* 2015; Donohue *et al.,* 2019; Greene *et al.,* 2021; Greene, Clayton, et al., 2019b; Trosvik *et al.,* 2018; Umanets *et al.,* 2018). Experimental studies in captive populations, and in humans, have shown that gut microbiomes can rapidly respond to changes in host diet (David *et al.,* 2014; Greene *et al.,* 2018), likely in species-specific ways. Taken together, these studies provide growing indication that the primate gut microbiome can promote host nutrition under diverse dietary conditions. We stand to benefit from greater comparative research to probe how the gut microbiomes of species with diverse feeding strategies differently facilitate host success under changing and challenging dietary conditions.

The lemurs of Madagascar comprise a powerful, nonmodel system in which to explore the links between feeding strategies, resource availability, and primate gut microbiomes. Lemurs are both phylogenetically and ecologically diverse. Many lemur genera are highly speciose, with members variably living in allopatry or sympatry across Madagascar's forest ecosystems. Across lineages and habitats, lemurs cope with Madagascar's hypervariable climate and environments via numerous evolutionary and ecological strategies (Dewar & Richard, 2007; Ganzhorn, 1988; Wright, 1999), and in some cases, distant relatives converged on similar strategies to solve similar problems. Most Malagasy forests are home to multiple lemur species from across the lemuriform clade that are characterized by both unique and convergent ecological traits.

In the present study, our focal species are Danfoss' mouse lemur (*Microcebus danfossi*, family Cheirogaleidae), the common brown lemur (*Eulemur fulvus*, family Lemuridae), the Coquerel's sifaka (*Propithecus coquereli*, family Indriidae), and the Anjiamangirana sportive lemur (*Lepilemur grewcockorum*, family Lepilemuridae) (Fig. 1a). These species live sympatrically in the Anjajavy Forest, which is located on Madagascar's northwest coast and boasts >10,000 ha of protected land. The site is dominated by dry deciduous forests, limestone or "tsingy" forests, and mangrove forests interspersed with abandoned agricultural land in various stages of recovery. Lemurs are among Earth's most endangered mammals (IUCN, 2021) but are safeguarded at Anjajavy through a combination of cultural practices, called "fady," that inhibit hunting and through commitments from local parties to sustainable ecotourism and conservation.

Our focal species belong to phylogenetic families that rapidly diverged *ca*. 40 million yr ago (Dos Reis *et al.,* 2018); however, the exact branching patterns remain a matter of ongoing debate (Marciniak *et al.,* 2021), limiting their utility in host–microbiome phylosymbiotic studies. Nevertheless, our focal species are characterized by diverse feeding strategies and differentially cope with food scarcity in the extreme dry seasons of Madagascar's northwest. This region naturally experiences weather extremes, including a yearly dry season when fruits, flowers, and potable water are scarce. As nocturnal omnivores, mouse lemurs in the northwest fallback on tree gums, insect exudates (Joly-Radko & Zimmermann, 2010; Thorén *et al.,* 2011) and nymphs (HAR, *pers. obs.*), and cockroaches foraged from the forest floor (ER, *pers. obs.*). As cathemeral frugivores, brown lemurs in the northwest forage overnight to reduce overall energy expenditure and fallback on some leaves during the dry season, which might serve nutritional and rehydration functions (Sato *et al.,* 2014). As diurnal frugo-folivores, sifakas consume a greater abundance of leaves and bark in times of fruit and flower scarcity (Sato *et al.,* 2016). Lastly, as nocturnal folivores, sportive lemurs readily

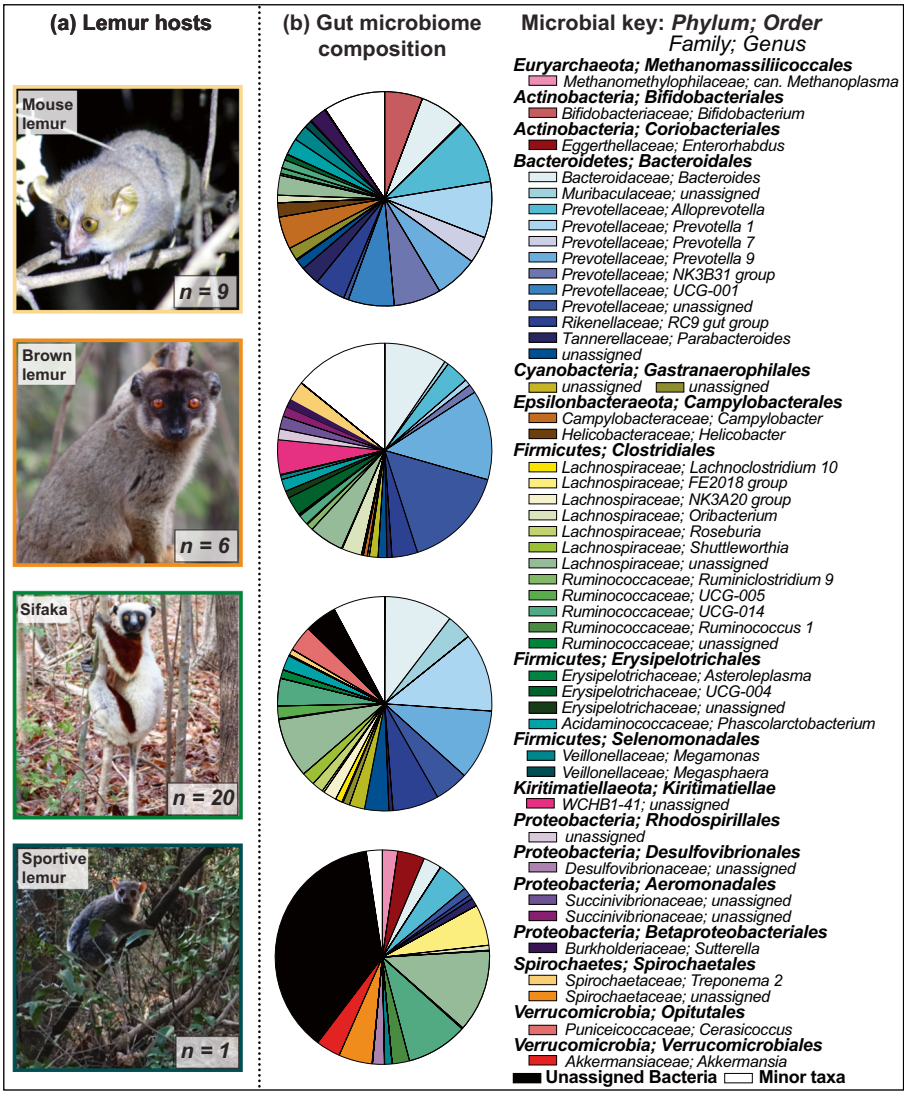

Fig. 1 Gut microbiome membership in four lemur species living in Anjajavy Forest, Madagascar. Shown are (**a**) photographs of a mouse lemur, brown lemur, sifaka, and sportive lemur at this site and (**b**) pie charts detailing the major microbial genera that accounted for >1% of the total microbiome, when calculating means across conspecifics, in minimally one species. Across pie charts, color families reflect microbial phyla and distinct shades reflect distinct genera. Taxon phylum, order, family, and genus identity are provided in the key. "Minor taxa" refers to the summation of all genera that failed to reach the 1% cutoff. "Unassigned" refers to the summation of all genera that could not be phylogenetically assigned below the specified level.

eat mature leaves and can subsist on some of the toughest diets recorded for primates (Hladik & Charles-Dominique, 1974; Thalmann, 2001).

Anjajavy's diverse assemblage of endemic lemurs provides a natural experiment for comparative microbiome research, while controlling for concurrent environmental variability. We therefore collected fecal samples from mouse lemurs, brown lemurs,

sifakas, and a single sportive lemur during the dry season and used amplicon sequencing to characterize and compare gut microbiome structure across our target species. We first ask how similar or dissimilar microbiome composition is across these distantly related host species: If feeding strategies— intertwined with host phylogeny—shape primate gut microbiomes across evolutionary time, we predict the host species will harbor unique gut microbial assemblages despite living in sympatry. We next ask if Anjajavy's sifakas and sportive lemur—species that independently acquired folivorous feeding strategies—share convergent gut microbial features: If folivory can be facilitated by different microbiome compositions, as has been shown in other Malagasy forests (Greene *et al.,* 2020; Perofsky *et al.,* 2019), we predict the sifakas and sportive lemur to harbor largely dissimilar gut microbiomes. We last examine our focal species' gut microbiomes in the context of fallback-food ecology: If lemur gut microbiotas perform metabolic functions that differentially enable their hosts to survive on digestibly challenging diets in lean periods, we predict mouse lemur microbiomes in the dry season to show signatures of gum fiber or chitin digestion, brown lemur microbiomes to show the greatest signatures of simple fiber metabolism, and sifaka and sportive lemur microbiomes to respectively show different signatures of recalcitrant fiber and plant-secondary compound metabolism.

Methods

Study Site, Subjects, and Sampling

Our study site was a private reserve in Anjajavy Forest, northwest Madagascar. Our subjects were 36 adult and subadult lemurs, of which nine were mouse lemurs (5 females; 4 males), 6 were brown lemurs (3 females; 2 males; 1 of unknown sex), 20 were sifakas (11 females; 8 males; 1 of unknown sex), and 1 was a sportive lemur (1 female). We collected a single, fresh fecal sample per subject, following defecation. We sampled lemurs in the middle of the dry season (July–August) in 2017 (mouse lemurs, brown lemurs, and sifakas) and 2018 (mouse lemurs, sifakas, and the sportive lemur).

We sampled habituated brown lemurs and sifakas along established trails, during walks in different areas on different days. We noted group composition and unique animal markings, so as to avoid resampling individuals. We collected samples from mouse lemurs captured via Sherman traps placed along established trails (Andriambeloson *et al.,* 2020; Blanco *et al.,* 2020). During handling, we gave mouse lemurs a unique transponder chip, enabling us to avoid resampling individuals. The single sportive lemur was serendipitously recovered from her sleeping site inside a tree hole, when we mistook the animal for a radiocollared dwarf lemur hibernating nearby. Between recovery and subsequent release, this individual defecated.

We scooped all fecal samples into sterile tubes, immediately submerged them in microbiome buffer (OMNIgene.GUT, DNA Genotek, Ottawa, Canada) (Brown *et al.,* 2018) and placed them out of direct sunlight at ambient temperature. We maintained these conditions during transit to Duke University, where samples were stored at −80°C until analysis. This methodology was previously shown to accurately preserve lemur gut microbiome composition (Greene *et al.,* 2021).

Amplicon Sequencing, Bioinformatics, and Statistics

We extracted genomic DNA using Qiagen's DNeasy PowerSoil Kit following an established workflow (Greene *et al.,* 2020; McKenney *et al.,* 2017). We shipped extracted samples (12-μL aliquots; 5–50 ng/μL) to Argonne National Laboratories (Lemont, IL) for amplicon sequencing. We targeted the V4 variable region of the microbial 16S rRNA gene using the 515f and 806r primers, 150 × 150 bp paired-end reads, and Illumina's MiSeq platform.

We processed reads using an established bioinformatics pipeline (Greene *et al.,* 2020), implemented in the Quantitative Insights Into Microbial Ecology software (QIIME 2; versions 2019.4 and 2020.6) (Bolyen *et al.,* 2019). In brief, paired-end reads were joined, demultiplexed, and filtered to remove low-quality, chimeric, and singleton reads. Using this regimen, we retained minimally 43,000 high-quality, raw sequences per sample. We binned these reads into amplicon sequence variants (ASVs) based on 100% sequence identity. We removed any ASVs present in only one sample, except for those singularly present in the sportive lemur.

We assigned taxonomy to ASVs using a *de novo* trained naïve Bayes classifier built from reads extracted for the 515–806 region from the SILVA 132 database (Quast *et al.,* 2012). We filtered out chloroplast and mitochondrial sequences. We collapsed our ASVs at phylum and genus level resolution for analysis. We calculated the mean relative abundance of microbial phyla and genera across conspecifics. Next, we determined the microbial genera that were present in any or all members of one host species or that were shared by any or all members across multiple host species. We focused on microbial taxa that were ubiquitously present (or absent) across conspecifics for the sake of simplicity. From the subset of shared microbes, we determined those genera that were significantly enriched in particular host species via linear discriminant analysis effect size (LEfSe) (Segata *et al.,* 2011), followed by conservative correction for multiple testing (Benjamini & Hochberg, 1995).

From the full dataset, we calculated metrics of community α and β diversity, rarefying to 25,000 reads/sample at the time of metric computation. For α diversity, we determined Observed Features, the Shannon index, and Faith's Phylogenetic Diversity, which respectively capture community richness, evenness, and phylogenetic breadth. These metrics were normally distributed. Across all statistical analyses, we excluded data from the sportive lemur, but report this individual's values in the figures, for comparative purposes. We performed three analyses of variance (ANOVAs) using the aov function in R Studio (version 1.3.959) (RStudio Team, 2020) and R software (version 4.0.2) (R Core Team, 2020), in which we entered each metric of α diversity as the response variable and the three main host species as the explanatory variable. We used Tukey's *post hoc* tests to determine significant pairwise comparisons. We reran the analyses using sex as an additional explanatory variable, but because these results were not statistically significant, we present the results from the simpler models.

For β diversity, we computed unweighted and weighted UniFrac distances, which capture the proportion of unique taxa between two samples, with the former reflecting the presence or absence of shared taxa and the latter reflecting their relative abundances (Lozupone *et al.,* 2011). We performed permutational multivariate ANOVAs using distance "adonis" analysis in QIIME 2, in which we entered UniFrac distances as the response variable and the three main host species as the explanatory variable. We reran

these analyses using sex as an additional explanatory factor. Because these results were not statistically significant, we report the results from the simpler model.

Next, we retained pairwise comparisons of β diversity for which the samples either derived from two conspecifics or from two members of different species. We used intraspecific, pairwise values to test for species differences in community variability among individuals and interspecific, pairwise values to determine which of the three main species harbored similar consortia. For both sets of analyses, we used Kruskal–Wallis and Dunn's *post hoc* tests implemented in GraphPad Prism (version 8.4.3). To check that differences in group membership (for sifakas and brown lemurs) or sampling location (for mouse lemurs) did not bias our results, we repeated the analysis of intraspecific variability by removing any comparisons deriving from two individuals sampled in the same group or captured in the same area. Because these repeat analyses produced nearly identical results to the full analyses, we report the findings from the full dataset.

Lastly, we ran sequences through the PICRUSt2 software to predict metagenomic function from microbial identities (Douglas *et al.,* 2020). Because many microbes in our dataset were unassigned at lower-order taxonomic ranks, with a bias against the folivores, we mapped only *ca.* 5% of our ASVs, which returned only a few hundred pathways. Given that these results are not fully representative of the metabolic processes occurring in focal species' microbiomes, we do not report these results.

Ethical Note

This research was approved by Duke University's IACUC (protocol A263-17-12) and by the Government of Madagascar's Ministry of Environment, Ecology and Forestry (permit numbers 136/17 and 035/18 MEEF/SG/DGF/DSAP/SCB.Re). The authors declare we have no conflict of interest.

Data Availability Raw sequence reads and associated metadata are available on the NCBI SRA under accession numbers PRJNA495032 and PRJNA684050 (sifakas and brown lemurs) and PRJNA757756 (mouse lemurs and the sportive lemur). Our bioinformatics pipeline is available online (Greene *et al.,* 2020).

Results

Each lemur species harbored a compositionally distinct gut microbiome across taxonomic levels (Fig. 1). At the phylum level, the consortia of all lemurs were dominated by *Bacteroidetes* and *Firmicutes* but in varying percentages. *Bacteroidetes* were most prevalent in mouse lemurs, accounting for 61% of the microbiome, were similarly abundant in brown lemurs and sifakas, respectively accounting for 52% and 53% of the microbiome, and were least abundant in the sportive lemur, accounting for only 11% of this individual's microbiome. *Firmicutes* displayed the opposite pattern of prevalence, accounting for only 18% of the microbiome in mouse lemurs, 26% and 29% of the microbiome in brown lemurs and sifakas, respectively, and 34% of the microbiome in

the sportive lemur. Other bacterial phyla, such as *Actinobacteria*, *Epsilonbacteraeota*, *Kiritimatiellaeota*, *Proteobacteria*, and *Verrucomicrobia*, were also variably abundant across host species. The *Synergistetes* phylum was uniquely absent from the consortia of all mouse lemurs in the study, but was present in all other individual lemurs, accounting for mean values of 0.1%–0.4% of the microbiome in the other species. The sifakas and sportive lemur, but not the other species, prevalently harbored unassigned bacteria, i.e., taxa that could not be identified below domain membership via online databases. These unassignable taxa accounted for only 5% of sequences in sifakas but for fully 37% of the sportive lemur's microbiome. Lastly, the *Euryarchaeota* phylum from the Archaea domain was most abundant in the sportive lemur's consortia.

Host species differed in the presence and relative abundance of microbial taxa at the genus level. Overall, we identified minimally 185 genera present in more than one individual lemur. Only six (3%) of the identified genera were present in 100% of individuals in the study; these included *Collinsella*, *Bacteroides*, *Prevotella 1*, *Parabacteroides*, the *RC9 gut group* from the Rikenellaceae family, and *UCG-014* from the Ruminococcaceae family (Fig. 2). Two more genera, *UCG-001* from the Prevotellaceae family and *Phascolarctobacterium*, were present in all individuals save the sportive lemur. Unassigned microbes from within the Prevotellaceae and Lachnospiraceae families were also shared among lemurs in the study; however, the ubiquity of these taxa should be interpreted with caution, as these groupings collapse all sequences that could not be identified below family-level membership and may represent multiple genera.

Of the microbial taxa variably present across lemurs, 76 genera were unique to one host species; 24 of these 76 were ubiquitously present among individuals for that host species (Fig. 2). For example, five microbial genera (*Bifidobacterium, Odoribacter*, *Blautia*, *Peptococcus*, and a genus within the Muribaculaceae family) were unique to mouse lemurs and present in all nine subjects. Eleven genera (e.g., a suite of Lachnospiraceae taxa, *Anaerovibrio*, and an unidentified member of the *S*uccinivibrionaceae family) were unique to brown lemurs and present in all six subjects. Eight taxa (*Lachnoclostridium 10*, *Anaerofilum*, *Mailhella*, *Succinivibrio*, UCG-011 from the Defluviitaleaceae family, and unassigned taxa from the Lachnospiraceae and Burkholderiaceae families and Coriobacteriales order) were unique to sifakas and present in all 20 subjects. In addition, one genus, *Shuttleworthia*, was common to all sifakas and to the sportive lemur. Of the microbial genera shared across species, LEfSe analysis revealed 74 to be significantly enriched in a single species (Fig. 3). We noted significant tradeoffs between host species in the relative abundances of genera from the Prevotellaceae family (*Bacteroidetes*) and the Lachnospiraceae, Ruminococcaceae, and Erysipelotrichaceae families (*Firmicutes*). Other major differences included that mouse lemurs harbored significant *Campylobacter* and *Helicobacter* (*Epsilonbacteraeota*), brown lemurs harbored abundant *Treponema2* (*Spirochaetes*) and an unassigned genus from the *Kiritimatiellaeota* phylum, and sifakas harbored abundant Christensenellaceae (*Firmicutes*), *Fibrobacter* (*Fibrobacteres*), and *Cerasicoccus* (*Verrucomicrobia*) phylum. In sum, most of the microbes we identified either were present in only one host species or were significantly more abundant in one host species relative to the others.

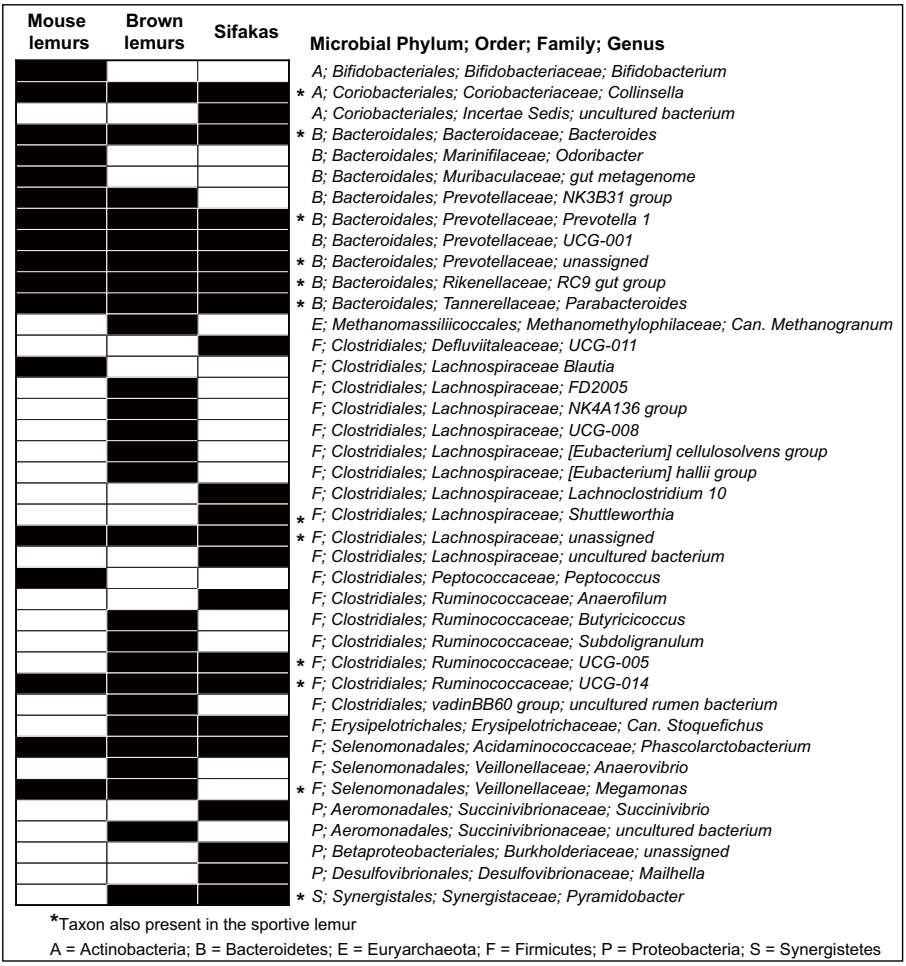

Fig. 2 The gut microbial members that are ubiquitously present or absent across sympatric mouse lemurs, brown lemurs, and sifakas in Anjajavy Forest, Madagascar. Rows represent distinct microbial genera, with the exception of the three "unassigned" genera that represent all confamiliar taxa unidentifiable at genus-level resolution. Higher-order taxonomy is provided in the text. Columns represent lemur species. Black rectangles indicate those taxa that are present in 100% of individuals within a host species, whereas white rectangles indicate those taxa that were present in 0% of individuals within a host species. Stars indicate the microbes that were also present in the sportive lemur's microbiota.

We found significant differences in α diversity across host species, as captured by Observed Features (ANOVA: $F_{2,32} = 36.45$, $P < 0.001$; Fig. 4a), the Shannon index (ANOVA: $F_{2,32} = 13.58$, $P < 0.001$; Fig. 4b), and Phylogenetic Diversity (ANOVA: $F_{2,32} = 22.65$, $P < 0.001$; Fig. 4c). For Observed Features, *post hoc* pairwise comparisons revealed that brown lemurs had the richest consortia, and sifakas had richer consortia than did mouse lemurs ($P < 0.001$ for all comparisons; Fig. 4a). For the Shannon index, *post hoc* comparisons showed that the consortia of brown lemurs and sifakas had equivalent evenness ($P = 0.3483$), exceeding that of mouse lemurs ($P < 0.001$ for both comparisons; Fig. 4b). For Phylogenetic Diversity, brown lemurs had consortia with greater scores compared to those of either sifakas or mouse lemurs ($P < 0.001$ for both comparisons; Fig.

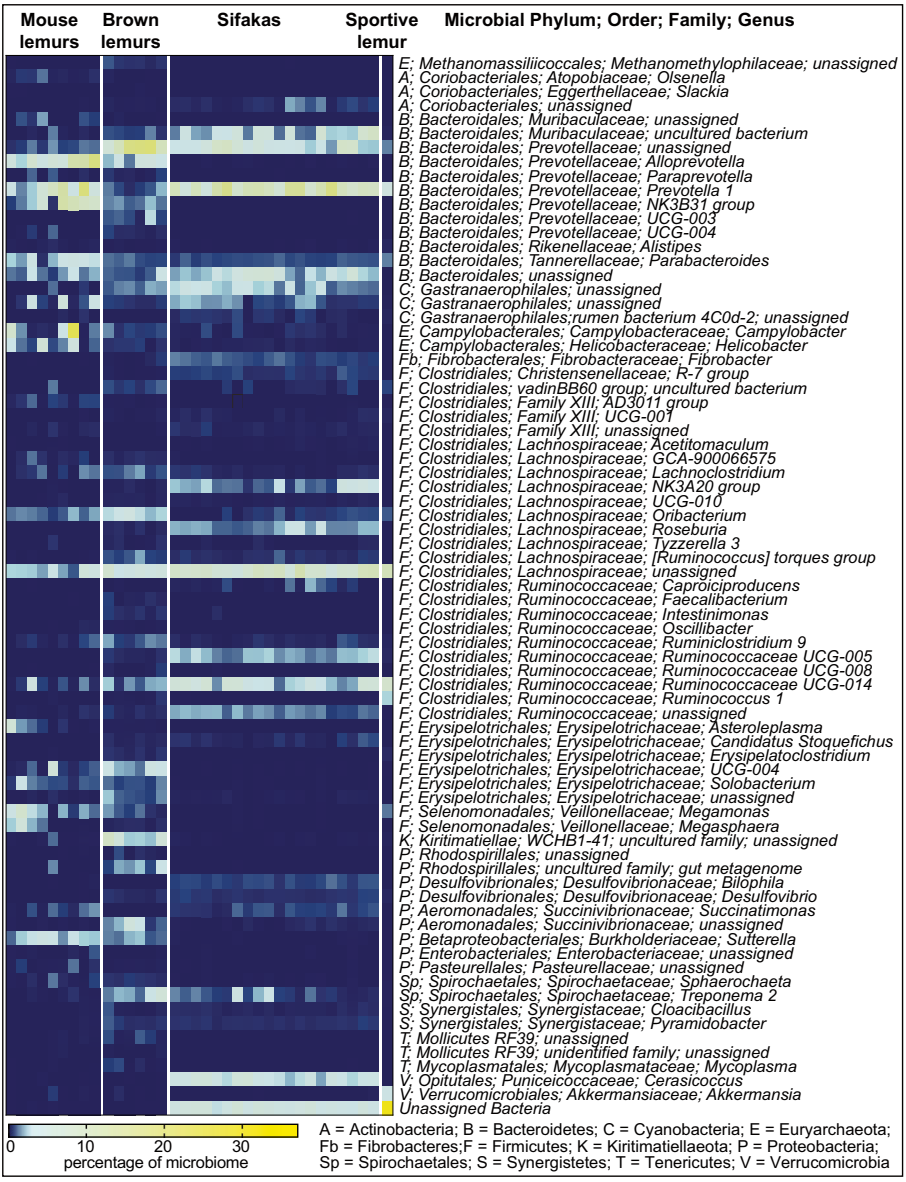

Fig. 3 Heatmap depicting the microbial groups that were significantly enriched in the gut consortia of mouse lemurs, brown lemurs, or sifakas in Anjajavy Forest, Madagascar. Rows represent distinct microbes, with taxonomic phylum, order, family, and genus membership provided in the text. Columns represent individual lemurs grouped by host species. Colors depict the relative abundance of each microbe on a continuous scale from dark blue (0%) to bright yellow (40%). Data from the sportive lemur were excluded from statistical analyses but are included in the figure for comparative purposes.

4c), whereas the consortia of sifakas and mouse lemurs had equivalent scores (P = 0.1477). Overall, brown lemur consortia were consistently the most diverse. Although not included in the statistical analyses, the consortia of the sportive lemur had scores of

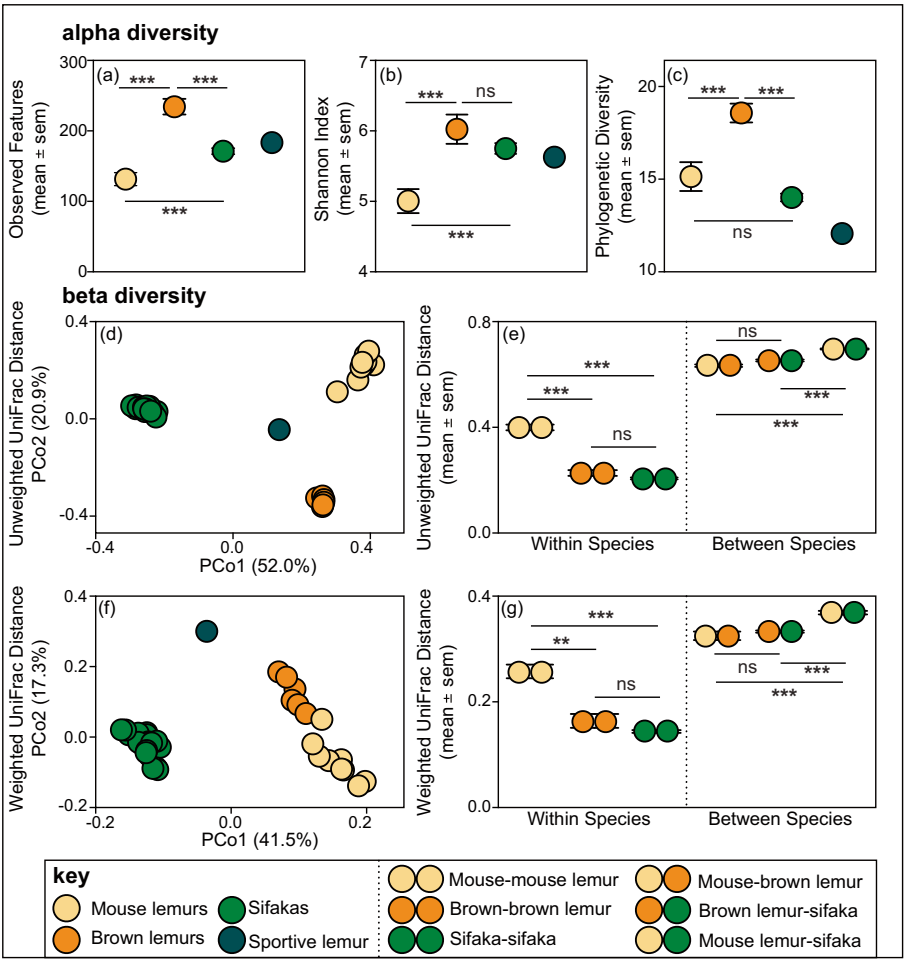

Fig. 4 Diversity in the gut microbiome of four lemur species in Anjajavy Forest, Madagascar. Shown are metrics of α diversity, including **(a)** Observed Features, **(b)** the Shannon index, and **(c)** Faith's Phylogenetic Diversity, and of ß diversity, including **(d, e)** unweighted and **(f, g)** weighted UniFrac Distances. UniFrac distances are plotted **(d, f)** in principal coordinate space and **(e, g)** as pairwise comparisons in which the individual lemurs derived from the same or different host species. Colors represent distinct host species, including mouse lemurs (tan), brown lemurs (orange), sifakas (green), and the sportive lemur (teal). We excluded data from the sportive lemur from statistical analyses but included this individual's values in the figure for comparative purposes. $^{**}P < 0.01$; $^{***}P < 0.001$; ns $P > 0.05$.

Observed Features and the Shannon index that were on par with those of the sifakas, but the score for Phylogenetic Diversity was, by far, the lowest in the study.

Lastly, we found β diversity to vary by host species, such that microbiome composition was more similar within than between species (Fig. 4d–g), as captured by unweighted (PERMANOVA: $R^2 = 0.78$, $F = 55.35$, $P < 0.001$; Fig. 4d) and weighted (PERMANOVA: $R^2 = 0.62$, $F = 26.27$, $P < 0.001$; Fig. 4f) UniFrac distances. Pairwise comparisons of conspecific β diversity further showed that variation among individuals differed between species (unweighted UniFrac: $H = 94.14$, $P < 0.001$, Fig. 4e; weighted UniFrac: $H = 68.0$, $P < 0.001$, Fig. 4g). *Post hoc* tests confirmed that mouse lemurs

harbored the most variable consortia compared to brown lemurs and sifakas (unweighted UniFrac: $P < 0.001$ for both comparisons, Fig. 4e; weighted UniFrac: $P < 0.01$ for both comparisons, Fig. 4g). Pairwise comparisons across species also varied significantly (unweighted UniFrac: $H = 181.2$, $P < 0.001$, Fig. 4e; weighted UniFrac: $H = 87.65$; $P < 0.001$, Fig. 4g), indicating that some species harbored more or less similar consortia to each other. *Post hoc* tests revealed these results to be largely driven by dissimilarities between the consortia of mouse lemurs and sifakas, which were larger than the dissimilarities between the microbiomes of either species compared to brown lemurs ($P < 0.001$ for all comparisons).

Discussion

We compared the gut microbiomes of four, sympatric lemur species in northwest Madagascar during the dry season, a time when each host species relies on different fallback foods. In line with our first prediction, the lemurs' gut microbiomes were strongly species specific, and in particular, comprised unique microbial memberships. Of the hundreds of identified microbes, we found that only six bacterial genera were shared by all individuals examined in the study: These taxa could be prime candidates for future work addressing vertical mechanisms of microbial inheritance (Moeller *et al.,* 2018). In line with our second prediction, we found minimal support for convergent microbiome memberships between the folivorous sifakas and the sportive lemur. This pattern recapitulates findings from elsewhere in Madagascar (Greene *et al.,* 2020; Perofsky *et al.,* 2019) and across primates globally (Amato *et al.,* 2019) and suggests that these distant relatives solve the digestive challenges of folivory by harboring distinct gut microbiomes. In line with our final prediction, many of the differences we report in microbiome diversity, variability, and membership can be putatively linked to ecological variability in fallback foods. We devote much of our ensuing discussion to exploring these potential associations. Ultimately, the strength of these species differences supports a role for host feeding strategy, intertwined with host phylogeny, in modulating gut microbiome structure across lineages (Amato *et al.,* 2019; Greene, Bornbusch, *et al.,* 2019a; Ley *et al.,* 2008; Nishida & Ochman, 2018; Rojas *et al.,* 2021), with consortia being further shaped within lineages to help hosts cope with their specific ecological challenges (Donohue *et al.,* 2019; Greene, Clayton, *et al.,* 2019b; Grond *et al.,* 2020; Umanets *et al.,* 2018).

Most of the microbes identified in this study varied consistently by host species, either in presence or relative abundance. In general, mouse lemur consortia were characterized by abundant *Bifidobacterium* and numerous Prevotellaceae members; brown lemur and sifaka consortia comprised relatively similar abundances of *Bacteroidetes* and *Firmicutes*, but the dominant families and genera differed; and the sportive lemur harbored abundant *Firmicutes* and microbes that cannot yet be mapped to online databases. Although there are many reasons animals may harbor specific microbiotas, some of the patterns we report can be putatively linked to differences in feeding strategies and fallback foods. For example, northwestern mouse lemurs in the dry season rely on gums and insects (Joly-Radko & Zimmermann, 2010; Thorén *et al.,* 2011) and harbored consortia that appeared tuned to the digestion of these items: The *Bifidobacterium*, and to a lesser extent the *Odoribacter* genera, are

linked to gum metabolism in humans, model organisms, and/or experimental cultures (Alarifi *et al.,* 2018; Fu *et al.,* 2019, 2020; Wyatt *et al.,* 1986) and were unique to and omnipresent among the mouse lemurs. Likewise, the *Alloprevotella* and *Alistipes* genera, linked to gum digestion in mice (Fu *et al.,* 2019), were significantly enriched in the consortia of mouse lemurs relative to the other host lemurs. That gut microbes can readily ferment gum fibers into short-chain fatty acids (Fu *et al.,* 2020; Wang *et al.,* 2019), which are key nutrients for the host (Wong *et al.,* 2006), potentially explains how mouse lemurs meet their high energetic demands in lean seasons and harsh habitats. By contrast, the Lachnospiraceae and Ruminococcaceae families from the *Firmicutes* phylum, that are inversely correlated to gum digestion (Fu *et al.,* 2020) and known for their genetic capacity to ferment recalcitrant plant fibers (Biddle *et al.,* 2013), were minimally represented in the mouse lemur gut. Instead, *Blautia* and *Peptococcus*, the only two *Firmicutes* taxa that were unique to and omnipresent among the mouse lemurs, have been correlated to insectivory in other mammals (Delsuc *et al.,* 2014) and could be key to facilitating chitin digestion.

Unlike the mouse lemurs at Anjajavy, the brown lemurs, sifakas, and sportive lemur harbored consortia that seemingly reflected greater plant-fiber metabolism, as captured by the increased diversity and abundance of Lachnospiraceae and Ruminococcaceae taxa (Biddle *et al.,* 2013). The genera within these families varied by host species, in both their presence and relative abundances: For example, Ruminococcaceae *UCG-005*, which was absent from mouse lemur consortia, was enriched in the consortia of sifakas relative to those of brown lemurs. Echoing previous studies across Madagascar (Greene, Clayton, *et al.,* 2019b; Umanets *et al.,* 2018), these patterns are consistent with these latter species' shared reliance on fruits (that are more abundant in brown lemur diets) and leaves (that are more abundant in sifaka diets) (Sato *et al.,* 2016). Although cautioning against generalizations based on a single individual, the consortia of the sportive lemur appeared most tuned to leaf digestion. Notably, this individual showed the greatest abundance of *Firmicutes* members and archael methanogens, and harbored *Enterohabdus*, a genus within the Eggerthellaceae family. Also present in rainforest sportive lemurs (Greene *et al.,* 2020), members of the Eggerthellaceae family might play a specific role in flavonoid metabolism or plant-secondary compound metabolism more generally (Maruo *et al.,* 2008; Singh *et al.,* 2020).

Despite their reliance on dietary foliage, sifakas and the sportive lemur shared only a few similarities in their gut microbiota. The genus *Shuttleworthia* from the Lachnospiraceae family was the only microbe ubiquitously present in sifakas and the sportive lemur yet absent from the consortia of brown lemurs and mouse lemurs. Interestingly, *Shuttleworthia* is present in the gut microbiome of rainforest sifakas, but absent from that of rainforest sportive lemurs, indri, and woolly lemurs (Greene *et al.,* 2020), indicating that this taxon is not a universal marker of folivory in lemurs. Nevertheless, this genus increases in livestock fed diets rich in alfalfa flavonoids (Zhan *et al.,* 2017) and rapeseed meal (Onarman Umu *et al.,* 2018), suggesting a possible role for *Shuttleworthia* in facilitating plant fiber or secondary compound metabolism.

The folivorous lemurs in this study also shared an abundance of unassignable taxa, largely within the Lachnospiraceae family and bacterial domain. Although updates to online databases have improved taxonomic resolution, unassignable taxa remain common in the microbiota of folivorous lemurs, especially in the dry forests (Greene,

Bornbusch, *et al.,* 2019a; Perofsky *et al.,* 2019). The dynamic environmental change that occurred throughout lemur history as Madagascar migrated north (Dewar & Richard, 2012), coupled with the lack of nitrogen in endemic fruit (Donati *et al.,* 2017) and the unpredictable climates that characterize Madagascar today (Dewar & Richard, 2007), all contributed to the proliferation of folivorous lemurs and their associated microbiotas. That folivore diversity in Madagascar is mirrored by equally diverse microbes calls for additional research to characterize the wealth of unassigned taxa, the myriad metabolic functions that microbes may perform, and their role in lemur evolution.

Beyond microbial membership, we noted differences in overall microbiome diversity and variability that reflect differences in the focal species' ecology. Mouse lemurs showed highly variable consortia across individuals that could reflect high metabolic rates linked to their small body size (Aivelo *et al.,* 2016). Alternately, mouse lemurs are solitary foragers (although they can nest communally (Radespiel *et al.,* 2003)), which might lead to greater individual variation in microbiome features linked to variation in foraging behavior or environmental exposure. Mouse lemurs also had low richness and evenness scores, indicating that their consortia are dominated by fewer microbes present at relatively high distributions. Although reduced α diversity might simply be a hallmark of mouse lemur microbiomes, perhaps these more skewed gut communities reflect the digestion of seasonal fallback foods, such as gums and insect exudates (called honeydew) (Joly-Radko & Zimmermann, 2010; Thorén *et al.,* 2011), that require significant enrichment for few specialized taxa or metabolic functions. In contrast, the more diverse consortia of the brown lemurs could reflect their more generalized diets that comprise nutrients that are bioavailable to many microbes. Reduced α diversity has been linked to folivorous specialization in lemurs (Greene *et al.,* 2020; Greene, Clayton, et al., 2019b) and sloths (Dill-McFarland *et al.,* 2015) and might be a marker of real-time dietary specialization.

Consistent with this framework, microbial phylogenetic breadth, as captured by Phylogenetic Diversity, was greatest in brown lemurs relative to the other hosts. Thus, those species that presumably consumed more specialized diets at the time of sampling (i.e., mouse lemurs, sifakas, and the sportive lemur) harbored microbiomes comprising fewer microbial taxa from fewer microbial lineages. Most likely, these lineages are unique to each host species, as mouse lemurs and sifakas had the greatest dissimilarity scores between their consortia. Thus, despite often living in sympatry throughout their extant ranges, these lemurs diverge in ecological, morphological, and microbial traits.

By comparing gut microbiomes across lemurs in the dry season and the dry forests, we gain insights into the diverse metabolic responses that enable species to survive on different and challenging fallback foods under extreme conditions. Future studies could compare the results reported herein to similar species assemblages in other seasons and forests in Madagascar, most notably in the rainy season and rainforests. It would also be beneficial to compare gut microbiome features to foraging or dietary data across individual lemurs and species. Broader comparisons encompassing numerous host species, genera, and families could explicitly test for phylosymbiosis in lemur gut microbiomes (Greene, Bornbusch, et al., 2019a; Greene, Clayton, *et al.,* 2019b; Perofsky

et al., 2019). We unfortunately lacked the resolution to do so, as our focal lineages diverged rapidly with somewhat unresolved branching patterns (Marciniak *et al.,* 2021). Linked together, these various types of studies could help determine if consistent patterns emerge regarding microbiome structure, host–lineage affiliation, and feeding ecology under varying environmental pressures, ultimately contributing to our understanding of primate–microbial symbiotic evolution.

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