



The gut microbiome of Madagascar's lemurs from forest fragments in the central highlands

Lydia K. Greene^{1,2} · Tsinjo Andriatiavina³ · Elissa D. Foss¹ · Ando Andriantsalohimisantatra⁴ · Tahiry V. Rivoarison⁴ · Fenonirina Rakotoarison⁵ · Tiana Randriamboavonjy⁵ · Anne D. Yoder¹ · Fanomezana Ratsoavina³ · Marina B. Blanco^{1,2}

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Abstract

The gut microbiome is now understood to play essential roles in host nutrition and health and has become a dominant research focus in primatology. Over the past decade, research has clarified the evolutionary traits that govern gut microbiome structure across species and the ecological traits that further influence consortia within them. Nevertheless, we stand to gain resolution by sampling hosts in understudied habitats. We focus on the lemurs of Madagascar's central highlands. Madagascar's highlands have a deep history as heterogeneous grassland-forest mosaics, but due to significant anthropogenic modification, have long been overlooked as lemur habitat. We collected fecal samples from Verreaux's sifakas (*Propithecus verreauxi*), common brown lemurs (*Eulemur fulvus*), and Goodman's mouse lemurs (*Microcebus lehilahytsara*) inhabiting two protected areas in the highlands and used amplicon sequencing to determine gut microbiome diversity and membership. As expected, the lemurs harbored distinct gut consortia tuned to their feeding strategies. Mouse lemurs harbored abundant *Bifidobacterium* and *Alloprevotella* that are implicated in gum metabolism, sifakas harbored abundant *Lachnospiraceae* that are implicated in leaf-fiber metabolism, and brown lemurs harbored diverse consortia with abundant *WCBH1-41* that could be associated with frugivory in harsh seasons and habitats. Within brown lemurs, a suite of bacteria varied between seed-packed and leaf-packed feces, a proxy for dietary intakes, collected from the same group over days. Our results underscore the evolutionary and ecological factors that govern primate gut microbiomes. More broadly, we showcase the forests of Madagascar's central highlands as rich habitat for future research of lemur ecology and evolution.

Keywords Microbiota · Ecology · Sifaka · *Eulemur* · *Microcebus* · *Propithecus*

Introduction

The gut microbiome is intrinsically intertwined with host evolution and ecology (Clayton et al. 2018b; McFall-Ngai et al. 2013) and plays a particular role in mediating primate nutrition (Clayton et al. 2018a; Yadav et al. 2018). Gut

microbes are chiefly responsible for fermenting tough fibers, ingested from plants and insect exoskeletons, into bioavailable fuel (Delsuc et al. 2014; Den Besten et al. 2013). The gut microbiome also metabolizes plant defensive compounds (Kohl et al. 2014) and synthesizes vitamins, co-factors, and amino acids (Biesalski 2016; Newsome et al. 2020). Recent literature, relying on comparisons across host species, has clarified the factors that govern gut microbiome structure in primates, i.e., determined which microbes inhabit which species and perform what digestive functions. This body of work established that primate gut microbiomes largely reflect host phylogeny linked to feeding strategy and digestive physiology (Amato et al. 2019; Donohue et al. 2022; Greene et al. 2022b): More closely related hosts that share similar gut morphology and dietary regimens generally harbor more similar gut microbiomes. Within primate lineages or populations, microbiome variation is further tuned

✉ Lydia K. Greene
lydiagreene@gmail.com

¹ Department of Biology, Duke University, Durham, NC, USA

² Duke Lemur Center, Duke University, Durham, NC, USA

³ Department of Zoology and Animal Biodiversity, University of Antananarivo, Antananarivo, Madagascar

⁴ Ankafobe Protected Area, Missouri Botanical Gardens, Antananarivo, Madagascar

⁵ Royal Botanic Gardens Kew, Antananarivo, Madagascar

to environmental variables including diet, seasonality, and habitat (Barelli et al. 2020; Garber et al. 2019; Hicks et al. 2018).

Much of this information has derived from comparisons across species of wild lemurs, strepsirrhine primates from Madagascar. Lemurs are phylogenetically and ecologically diverse. Over 100 species belonging to 15 genera and 5 families are characterized by a variety of feeding strategies, and variably live in allopatry or sympatry across the island's many forested ecosystems (Mittermeier et al. 2023). Some species are ecologically specialized or geographically localized whereas others are more generalized and widely distributed. Most are listed as endangered or critically endangered on the IUCN Red List and face significant threats predominantly due to habitat loss and fragmentation (Mittermeier et al. 2023). To date, various lemur species and populations in the eastern rainforests (Donohue et al. 2019; Greene et al. 2020; Raulo et al. 2018), western dry forests (Greene et al. 2021; Murillo et al. 2022b; Perofsky et al. 2017), and southern spiny forests (Bornbusch et al. 2022; Wasimuddin et al. 2022) have contributed gut microbiome data to the literature. The lemurs of the mountainous central highlands remain wholly underrepresented.

Madagascar's central highlands are, however, a remarkable habitat for studies of lemur evolution, ecology, and adaptation. The highlands are the contiguous inland region that sit at 800–2000 m above sea level. Today, they form a mosaic of grasslands atop hills interspersed with forest fragments and agricultural fields in valleys. The highlands have been long overlooked as wildlife habitat owing, in large part, to the colonial narrative that the region was entirely forested prior to human arrival and is now almost completely anthropogenically degraded (Richard 2023; Silander et al. 2024). Even today, the history and becoming of the central highlands is a hotly disputed topic among the conservation and research community (Joseph et al. 2021). Emerging scholarship, however, points to a deep history of these biomes as mosaic and naturally fragmented, with the grasslands comprising both ancient endemics and more recently introduced species (Crowley et al. 2021; Silander et al. 2024; Vorontsova et al. 2016). The highlands historically have been rich, heterogeneous, and dynamic habitats that, at times, were connected to eastern and western forest blocks, serving as habitat corridors (Joseph et al. 2021; Yoder et al. 2016). A handful of established protected areas today conserve remnant forest fragments and inhabiting fauna, including lemurs.

The lemurs of the highlands today include six species—Goodman's mouse lemur (*Microcebus lehilahytsara*), the furry-eared dwarf lemur (*Cheirogaleus crossleyi*), the common brown lemur (*Eulemur fulvus*), and three species of sifaka (*Propithecus coronatus*, *P. deckenii*, and *P. verreauxi*) (Blanco et al. 2024a,b; Goodman et al. 2018; King et al. 2014; Rakotonirina et al. 2014). None is restricted to the

highlands: each species occupies other habitats in the eastern rainforests, western dry forests, and/or southern spiny forests. As a result, these species are among the most widely distributed and ecologically generalized of their respective genera. To survive in the highlands, lemurs variably face frigid conditions that impose thermoregulatory challenges and great exposure to forest edges. Many populations are likely required to navigate open grasslands to move between food patches and to disperse from natal groups. If the gut microbiome is part of the evolutionary and ecological toolkit that facilitates dietary and niche breadth (Greene et al. 2020), and thereby facilitates adaptation to local conditions—including naturally and anthropogenically changing landscapes—the highland lemurs are a critical system for study.

To demonstrate the feasibility of studying the gut microbiome in highland lemurs, we profiled consortia in three species inhabiting two protected areas during the core of the dry season (Fig. 1a–d). The first site, the Protected Harmonious Landscape of Itremo, sits at the geographic center of Madagascar. A vast and varied ecosystem, Itremo comprises pockets of sclerophyllous woodland, tapia (*Uapaca bojeri*), and invasive pine trees surrounded by grasslands along with numerous mid-altitude moist evergreen forest fragments within valleys. These fragments are home to a population of Verreaux's sifakas (Blanco et al. 2024b). The second site, the Ankafobe protected area, sits in the center of Madagascar's highlands and comprises just three tiny forest fragments totaling 33 ha surrounded by fire-prone grasslands. Ankafobe contains copious *Harungana madagascariensis* and *Pandanus* spp. and is one of the few sites to conserve the Sohisika tree (*Schizolaena tampoketsana*). Ankafobe's forest fragments are home to populations of common brown lemurs and Goodman's mouse lemurs. Both Ankafobe and Itremo are also home to populations of furry-eared dwarf lemurs (Blanco et al. 2018; 2024b), although, due to seasonal hibernation, are notably absent from our study.

Like all sifakas, Verreaux's sifakas are seasonal frugivores (Sato et al. 2016; Yamashita 2008): They boast the complex gut morphology (Campbell et al. 2000) and specialized gut microbiome to handle significant leaf intake (Greene et al. 2020, 2021; Springer et al. 2017). In contrast, brown lemurs are frugivores: although they eat leaf matter, particularly in the dry season (Sato et al. 2014, 2016), they have a relatively simple gastrointestinal tract (Campbell 2003) and generalized gut microbiome (Greene et al. 2021; Murillo et al. 2022a) to support fruit digestion. For both sifakas and brown lemurs, gut microbiome structure correlates to habitat occupancy (Greene et al. 2019; Umanets et al. 2018), as well as to seasonal consumption of fruits and leaves (Murillo et al. 2022a; Springer et al. 2017). Mouse lemurs have generally flexible diets of tree gums, insects, insect secretions, and fruits and flowers (Atsalis 1999;

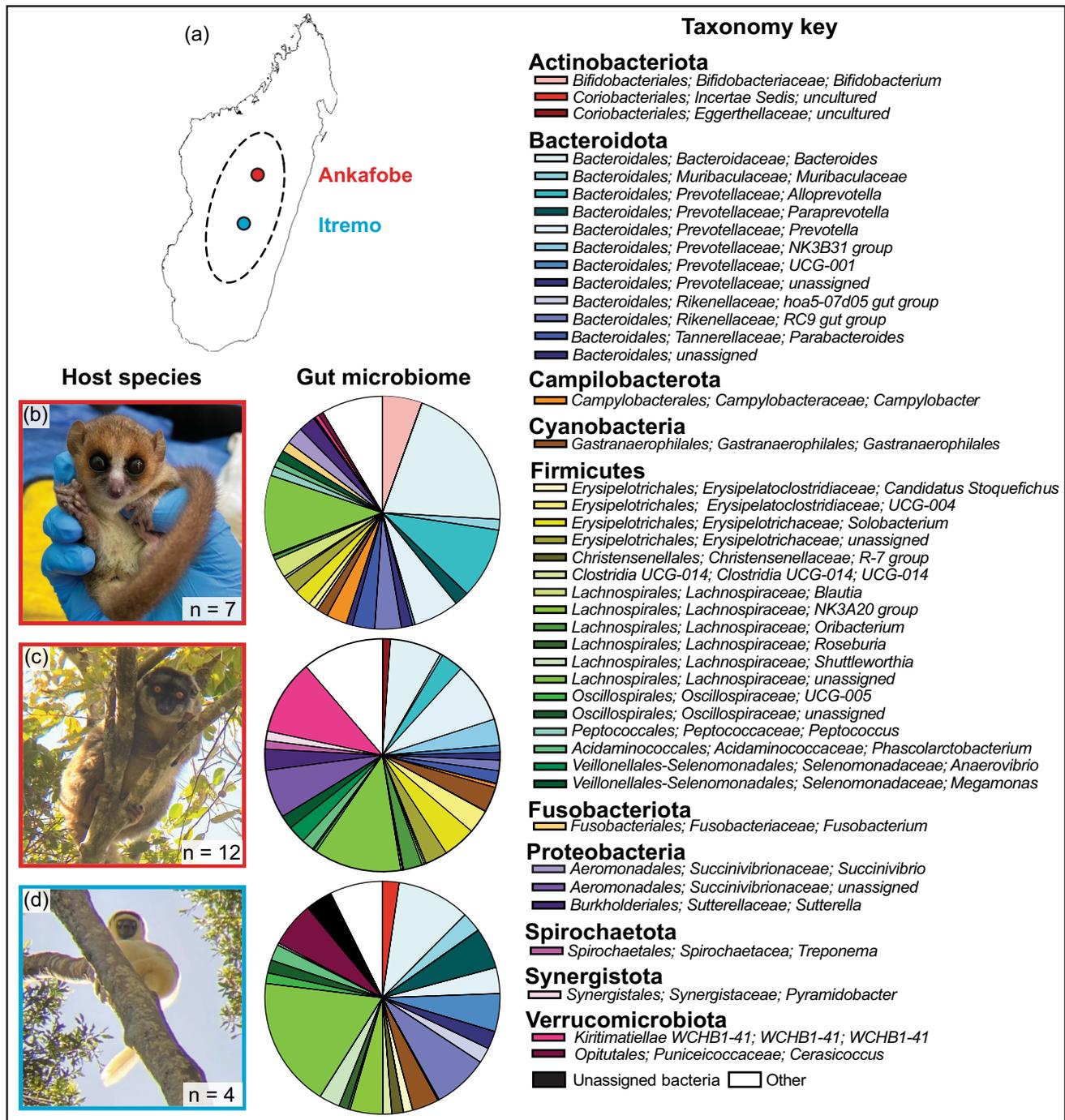


Fig. 1 a Map of Madagascar pinpointing the Itremo (blue) and the Ankafoabe (red) protected areas. The dotted line outlines the central highlands. Photos of the study species and pie charts depicting microbiome membership for **b** Goodman’s mouse lemurs, **c** common brown lemurs, and **d** Verreaux’s sifakas. Included in the pie charts

are all microbial genera that account for > 1% of the total microbiome in one species, when averaged across conspecifics. Color key for the taxa in the pie charts is provided to the right at phylum, order, family, and genus-level resolution where possible. White refers to the summation of all taxa that failed to reach the 1% cutoff

Dammhahn and Kappeler 2008; Radespiel et al. 2006). Like brown lemurs, mouse lemurs have a simple gastrointestinal tract (Campbell 2003), their gut microbiomes can reflect localized habitat or dietary intakes (Greene et al. 2021;

Wasimuddin et al. 2022), and can be widely variable across individuals (Aivelo et al. 2016). At Ankafoabe, mouse lemurs heavily rely on endemic mistletoes (*Bakerella clavata*) in

the dry season (Andriambelosen et al. 2021), in addition to gums and insects.

If host phylogeny tuned to digestive physiology and feeding ecology are primary drivers of gut microbiome structure in Malagasy lemurs (Amato et al. 2019; Donohue et al. 2022; Greene et al. 2020), we expect the populations of the central highlands to show patterns largely consistent with their congeners and conspecifics at other sites in Madagascar (Greene et al. 2021). We expect the mouse lemurs, brown lemurs, and sifakas of the highlands to host species-specific gut microbiomes. We further expect the consortia of brown lemurs to be the most diverse and generalized while those of mouse lemurs and sifakas will show signatures of their reliance on respective fallback foods in the dry season, including gums and insects for mouse lemurs and leaves for sifakas. Within the brown lemurs, our sampling depth further allows for exploration of how dietary fruits vs. leaves relate to gut microbiome features within the dry season. We expect leaf consumption to be associated with signatures of microbial specialization, i.e., increases in taxa known for their role in fiber fermentation and a concurrent reduction in diversity (Greene et al. 2020).

Methods

Sites, target species, and samples

During the dry season of 2022, our team traveled to Itremo to sample Verreaux's sifakas (June 11–29) (Fig. 1d) and to Ankafoabe to sample Goodman's mouse lemurs (Fig. 1b) and common brown lemurs (July 8–16) (Fig. 1c). The sites are approximately 280 km apart, but require 2–3 days by car to travel between them.

For sifakas and brown lemurs, we collected fecal samples during diurnal walks on established trails. At Itremo, sifakas are unhabituated to human presence, but the local guide team knew of two groups in different areas. On different days, the guide team located and quietly approached the groups and secured freshly voided fecal samples from three individuals in group 1 and from one individual in group 2.

At Ankafoabe, we encountered one semi-habituated social group of brown lemurs on 3 separate days. We secured 12 freshly voided fecal samples in total, including three samples from different individuals on day 1, five samples from different individuals on day 3, and four samples from different individuals on day 5. Because animals are not individually known in this population, it is likely that we repeatedly sampled some individuals on separate days. The samples contained a mixture of seed-packed ($n=9$) and leaf-packed ($n=3$) feces. One of us (T.V.R.) identified the seeds as

belonging to Sely (*Grewia* sp.), Harungana (*Harungana madagascariensis*), and Kafeala (*Coffea* sp.).

For mouse lemurs at Ankafoabe, we captured individuals in two forest fragments using standard live traps, baited with banana at sunset, and checked before sunrise (Andriambelosen et al. 2021). Captured individuals were brought to camp and mildly sedated (Ketamine, 10 mg/kg IM) to collect morphometric and reproductive data as part of our team's monitoring program on this population (Blanco et al. 2024a). During handling, mouse lemurs often defecate: we secured 9 freshly voided fecal samples during handling from 7 unique individuals, including 3 adult females, 3 adult males, and 1 juvenile female.

Fecal samples from all lemurs were immediately submerged in microbiome buffer (OMNIGene.GUT, DNA Genotek) and kept at room temperature out of direct sunlight until processing. Samples were transported to the genetics laboratory in the Department of Zoology and Animal Biodiversity (MZBA) at the University of Antananarivo for extraction. On July 23rd and 25th 2022, we extracted genomic DNA from samples using commercial kits (DNeasy PowerSoil Pro Kit, Qiagen). We followed kit protocols, except for heat-blocking prior to bead-beating and reducing the final volume of elution buffer to 70 μ L. All gDNA extracts were frozen at -20 °C. In August 2022, we aliquoted 30 μ L of each extract for exportation and sequencing. We left the remaining extract volumes for banking at the MZBA. We placed 30 μ L aliquots on icepacks in transport coolers and hand-carried them to Duke University, where they were frozen at -80 °C until sequencing.

Sequencing and bioinformatics

We shipped sample extracts to the Argonne National Laboratory (Lemont, IL) for library preparation and amplicon sequencing. We targeted the V4 region of the 16S rRNA gene using the 515f and 806r primers, 150 \times 150 bp paired-end reads, and Illumina's MiSeq platform.

We processed raw reads using an established bioinformatics pipeline implemented in the Quantitative Insights Into Microbial Ecology 2 (QIIME 2) software (version 2022.8) (Bolyen et al. 2019). We joined, demultiplexed, and filtered paired-end sequences to remove singleton, low-quality, and chimeric reads. Each sample was represented by between 9,430 and 60,581 high-quality, raw sequence reads. We binned sequences in Amplicon Sequence Variants (ASVs) based on 100% identity and removed ASVs present in only 1 sample. We assigned taxonomy to ASVs using the pre-trained classifier for the SILVA 138 database extracted for the 515–806 region (Quast et al. 2012). We removed mitochondrial and chloroplast sequences. We collapsed our ASVs at genus-level resolution (L6) for analyses. Lastly, we computed three metrics of alpha diversity, namely richness (observed features), evenness (the

Shannon index), and phylogenetic breadth (Faith's phylogenetic diversity), and two metrics of beta diversity, namely unweighted and weighted UniFrac distances. We rarefied to a depth of 8,000 reads/sample at the time of diversity–metric computation.

Statistics

We focused our statistical analyses on microbiome differences between host species, and within brown lemurs relative to fecal content (i.e., leaf vs. seed-packed samples). For two individual mouse lemurs, we secured two fecal samples: for statistics, we averaged values of taxonomy and diversity across repeat samples to include each individual lemur only once.

For comparisons across host species, we included data from mouse lemurs ($n = 7$), brown lemurs ($n = 12$), and sifakas ($n = 4$). First, we computed the relative abundance of each microbial genus per sample. We determined the microbial taxa that were unique to one host species: from the microbes shared across host species, we used Linear Discriminant Analysis Effect Size (LEfSe) to determine the genera significantly more abundant in one host species vs. the others (Segata et al. 2011). The three metrics of alpha diversity were all normally distributed: we performed a one-way analysis of variance (ANOVA) with Tukey's post hoc tests per metric using host species as the explanatory variable. We implemented these analyses in the Rstudio program (version 2023.12.0+369) (RStudio Team 2023) with R software (version 4.2.1) (R Core Team 2022). For the two metrics of beta diversity, we performed a permutational analysis of variance using distance matrices (adonis2) per metric, with host species as the explanatory variable. We implemented the adonis analyses using the vegan package (version 2.6–4) (Oksanen et al. 2022) and computed post hoc pairwise comparisons using the pairwiseAdonis package (version 0.4.1) (Arbizu et al. 2017).

We ran a similar analytical pipeline on only the subset of samples from the brown lemurs. We used LEfSe to determine the microbial genera that were significantly more abundant in leaf-packed ($n = 3$) vs. seed-packed samples ($n = 9$). We compared metrics of alpha and beta diversity to fecal content, respectively, using non-parametric Wilcoxon tests and adonis analysis.

Results

Microbiome variation between host species

We found strong differences in gut microbiome composition between the mouse lemurs, brown lemurs, and sifakas of Madagascar's central highlands (Fig. 1). At phylum-level

resolution, the gut microbiome of mouse lemurs ($n = 7$) was dominated by Bacteroidota (50.4%), Firmicutes (28.7%), Actinobacteriota (6.3%), Proteobacteria (5.3%), and Campylobacterota (3.4%); the gut microbiome of brown lemurs ($n = 12$) was dominated by Firmicutes (38.3%), Bacteroidota (29.2%), Verrucomicrobiota (11.1%), Proteobacteria (10.8%), and Cyanobacteria (3.5%); and the gut microbiome of sifakas ($n = 4$) was equally dominated by Bacteroidota (39.8%) and Firmicutes (39.7%), with lesser contributions from Verrucomicrobiota (6.3%), Actinobacteriota (4.0%), and Cyanobacteria (3.7%). Only the sifaka consortia contained bacterial sequences that could not be identified below domain-level resolution (3.7%). Below phylum-level resolution, we identified 150 taxa present in more than one fecal sample. Most were assignable to a microbial genus ($n = 138$) or family ($n = 7$).

Of these 150 taxa, we determined which were shared among hosts within and between host species. Only 12 microbial taxa were shared among 100% of samples in the study (Fig. 2). These common members include well-known genera like *Prevotella*, *Parabacteroides*, *Campylobacter*, and *Helicobacter*. Sympatric brown and mouse lemurs all shared a further four genera that were absent in sifakas, including *Megamonas* and *Sutterella*. Allopatric brown lemurs and sifakas all shared six genera that were absent in mouse lemurs, including *Enterorhabdus* and *Shuttleworthia*. No single taxon was present in all mouse lemur and sifaka samples but absent in brown lemur samples. All mouse lemurs shared six taxa that were absent in the other species, including *Odoribacter*, *Blautia*, and *Peptococcus*. All brown lemurs shared seven taxa absent in mouse lemurs and sifakas, including several members of the *Prevotellaceae* family. All sifakas shared six taxa absent in mouse and brown lemurs, including several members of the *Desulfovibrionaceae* family (Fig. 2). The remaining taxa showed variable patterns of presence and absence within and between hosts.

In total, we identified 83 taxa that were present in minimally two host species, although not necessarily in all samples. Of these, fully 61 were significantly more abundant in one host species compared to the others. 12 taxa were significantly more abundant in mouse lemur consortia, including *Bifidobacterium* ($\log(\text{LDA}) = 4.49$, $p < 0.001$), *Alloprevotella* ($\log(\text{LDA}) = 4.75$, $p = 0.001$), *Parabacteroides* ($\log(\text{LDA}) = 4.25$, $p = 0.002$), *Bacteroides* ($\log(\text{LDA}) = 4.85$, $p = 0.007$), and *Campylobacter* ($\log(\text{LDA}) = 4.17$, $p = 0.007$). A further 25 taxa were significantly more abundant in brown lemur consortia, including *Prevotella* ($\log(\text{LDA}) = 4.42$, $p = 0.03$), *Erysipelatoclostridiaceae* UCG-004 ($\log(\text{LDA}) = 4.31$, $p < 0.001$), *Solobacterium* ($\log(\text{LDA}) = 4.44$, $p = 0.001$), *Mycoplasma* ($\log(\text{LDA}) = 3.35$, $p < 0.001$), *Megamonas* ($\log(\text{LDA}) = 4.07$, $p = 0.007$), *Sutterella* ($\log(\text{LDA}) = 4.26$, $p = 0.005$), and *Kiritimatiellae* WCHB1-41 ($\log(\text{LDA}) = 4.79$, $p < 0.001$); and

Mouse lemurs	Brown lemurs	Sifakas	Microbial Phylum; Order; Family; Genus
			Domain: Archaea
			T; Methanomassiliicoccales; Methanomethylophilaceae; unassigned
			Domain: Bacteria
			A; Coriobacteriales; Eggerthellaceae; Enterorhabdus
			B; Bacteroidales; Bacteroidaceae; Bacteroides
			B; Bacteroidales; Marinifilaceae; Odoribacter
			B; Bacteroidales; Muribaculaceae; Muribaculaceae
			B; Bacteroidales; Prevotellaceae; Prevotella
			B; Bacteroidales; Prevotellaceae; NK3B31 group
			B; Bacteroidales; Prevotellaceae; UCG-003
			B; Bacteroidales; Prevotellaceae; UCG-004
			B; Bacteroidales; Rikenellaceae; hoa5-07d05 gut group
			B; Bacteroidales; Rikenellaceae; RC9 gut group
			B; Bacteroidales; Tanerellaceae; Parabacteroides
			Ca; Campylobacteriales; Campylobacteraceae; Campylobacter
			Ca; Campylobacteriales; Helicobacteraceae; Helicobacter
			Cy; Gastranaerophilales; Gastranaerophilales; Gastranaerophilales
			D; Desulfovibrionales; Desulfovibrionaceae; Mailhella
			D; Desulfovibrionales; Desulfovibrionaceae; unassigned
			Fi; Fibrobacteriales; Fibrobacteraceae; Fibrobacter
			F; Erysipelotrichales; Erysipelatoclostridiaceae; UCG-004
			F; Erysipelotrichales; Erysipelotrichaceae; Solobacterium
			F; Lachnospirales; Defluviitaleaceae; UCG-011
			F; Lachnospirales; Lachnospiraceae; [Eubacterium] hallii group
			F; Lachnospirales; Lachnospiraceae; Blautia
			F; Lachnospirales; Lachnospiraceae; Lachnoclostridium
			F; Lachnospirales; Lachnospiraceae; NK3A20 group
			F; Lachnospirales; Lachnospiraceae; UCG-008
			F; Lachnospirales; Lachnospiraceae; Shuttleworthia
			F; Lachnospirales; Lachnospiraceae; unassigned
			F; Oscillospirales; Oscillospiraceae; UCG-005
			F; Oscillospirales; Ruminococcaceae; Anaerofilum
			F; Peptococcales; Peptococcaceae; Peptococcus
			F; Acidaminococcales; Acidaminococcaceae; Phascolarctobacterium
			F; Veillonellales-Selenomonadales; Selenomonadaceae; Anaerivibrio
			F; Veillonellales-Selenomonadales; Selenomonadaceae; Megamonas
			F; Veillonellales-Selenomonadales; Veillonellaceae; Megasphaera
			Fu; Fusobacteriales; Fusobacteriaceae; Fusobacterium
			P; Aeromonadales; Succinivibrionaceae; Succinatimonas
			P; Aeromonadales; Succinivibrionaceae; Succinivibrio
			P; Aeromonadales; Succinivibrionaceae; unassigned
			P; Burkholderiales; Oxalobacteraceae; unassigned
			P; Burkholderiales; Sutterellaceae; Sutterella
			S; Synergistales; Synergistaceae; Cloacibacillus
			S; Synergistales; Synergistaceae; Pyramidobacter

Phyla: T = Thermoplasmatota; A = Actinobacteriota; B = Bacteroidota; Ca = Campilobacteria; Cy = Cyanobacteria; D = Desulfobacterota; Fi = Fibrobacterota; F = Firmicutes; Fu = Fusobacteriota; P = Proteobacteria; S = Synergistota

Fig. 2 Gut microbial taxa that are ubiquitously present or absent in the mouse lemurs, brown lemurs, and sifakas of the central highlands in Madagascar. Rows represent distinct microbial genera, except for five “unassigned” microbes that represent all confamilial taxa unidentifiable at genus-level resolution. Taxonomy at phylum, order, family, and genus are presented in the text beside each row. Columns represent lemur species. Colored 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

22 taxa were more abundant in sifaka consortia, including an uncultured *Coriobacteriales* genus (log(LDA)=4.11, $p < 0.001$), *Prevotellaceae* UCG-001 (log(LDA)=4.50, $p = 0.02$), *Rikenellaceae* RC9 group (log(LDA)=4.57, $p = 0.009$), *Cerasicoccus* (log(LDA)=4.58, $p < 0.001$), as well as *Shuttleworthia* (log(LDA)=4.24, $p < 0.001$), the *NK3A20* group (log(LDA)=4.43, $p < 0.001$), and unassigned members (log(LDA)=4.67, $p = 0.007$) all from the *Lachnospiraceae* family. For full results of differential abundance testing, see the supplementary material.

Beyond microbiome composition, alpha diversity varied significantly between host species, as characterized by metrics of richness (observed features: $F_{2,20} = 15.08$, $p < 0.001$; Fig. 3a), evenness (Shannon index: $F_{2,20} = 15.71$, $p < 0.001$; Fig. 3b), and phylogenetic breadth (Faith’s phylogenetic diversity: $F_{2,20} = 13.48$, $p < 0.001$; Fig. 3c). Post hoc tests highlighted that these species differences were largely driven by brown lemurs: The gut consortia of brown lemurs had greater scores of richness compared to those of mouse lemurs ($p < 0.001$) and sifakas ($p = 0.003$), as well as of evenness compared to both mouse lemurs ($p < 0.001$) and sifakas ($p < 0.001$), and of phylogenetic diversity compared to mouse lemurs ($p = 0.012$) and sifakas ($p < 0.001$). Mouse lemurs and sifakas had comparable scores of alpha diversity ($p > 0.12$ for all comparisons).

Beta diversity varied significantly across host species, as captured by the unweighted ($F_{2,20} = 41.5$, $R^2 = 0.81$, $p < 0.001$; Fig. 3d) and weighted ($F_{2,20} = 26.79$, $R^2 = 0.73$, $p < 0.001$; Fig. 3e) UniFrac metrics. Post hoc tests revealed differences between all three host species for the unweighted and weighted metrics ($p < 0.02$, for all pairwise comparisons).

Microbiome variation within brown lemurs relative to fecal content

Within the brown lemurs, we identified microbiome differences in seed-packed ($n = 9$) vs. leaf-packed ($n = 3$) samples. Although microbial presence did not differ between sample types, the relative abundance of 18 microbial taxa did (Fig. 4). Eight taxa were more abundant in leaf-packed samples, namely *Prevotella* (log(LDA)=4.13, $p = 0.02$; Fig. 4a), *Solobacterium* (log(LDA)=4.07, $p = 0.02$; Fig. 4b),

Eubacterium hallii group (log(LDA)=3.52, $p = 0.02$; Fig. 4c), *Lachnoclostridium* (log(LDA)=3.48, $p = 0.01$; Fig. 4d), *Lachnospiraceae* NK3A20 group (log(LDA)=3.36, $p = 0.03$; Fig. 4e), *Oribacterium* (log(LDA)=3.61, $p = 0.03$; Fig. 4f), *Megasphaera* (log(LDA)=3.84, $p = 0.01$; Fig. 4g), and unassigned members of the *Succinivibrionaceae* family (log(LDA)=4.35, $p = 0.02$; Fig. 4h).

In contrast, 10 taxa were enriched in seed-packed samples, namely *Alloprevotella* (log(LDA)=3.87, $p = 0.03$; Fig. 4i), *Prevotellaceae* UCG-004 (log(LDA)=3.44, $p = 0.01$; Fig. 4j), *Gastranaerophilales* (log(LDA)=3.91, $p = 0.03$; Fig. 4k), *Erysipelatoclostridium* (log(LDA)=3.08, $p = 0.02$; Fig. 4l), *Erysipelatoclostridiaceae* UCG-004 (log(LDA)=4.08, $p = 0.01$; Fig. 4m), *Clostridia* UCG-014 (log(LDA)=3.28, $p = 0.03$; Fig. 4n), *Clostridia* vadinBB60 group (log(LDA)=3.41, $p = 0.01$; Fig. 4o), uncultured *Rhodospirillales* (log(LDA)=3.53, $p = 0.01$; Fig. 4p), *Treponema* (log(LDA)=3.78, $p = 0.01$; Fig. 4q), and *Kiritimatiellae* WCHB1-41 (log(LDA)=4.40; $p = 0.03$; Fig. 4r).

Microbiome alpha diversity trended towards being greater in seed-packed vs. leaf-packed samples. Although richness did not differ by fecal content (observed features: $W = 5$, $p = 0.138$; Fig. 5a), seed-packed vs. leaf-packed samples trended towards having greater microbiome evenness and phylogenetic breadth ($W = 3$, $p = 0.063$, for both the Shannon index and Faith’s phylogenetic diversity; Fig. 5b,c). Beta diversity varied between seed-packed and leaf-packed samples, as captured by the unweighted ($F_{1,10} = 2.565$, $R^2 = 0.20$, $p = 0.02$; Fig. 5d) and weighted ($F_{1,10} = 4.251$, $R^2 = 0.30$, $p = 0.006$; Fig. 5e) UniFrac metrics.

Discussion

By profiling the gut microbiome of lemurs from Madagascar’s central highlands, we add new insights to our understanding of primate microbial ecology. Overall, we found predictable patterns of gut microbial membership across focal species that broadly match those from related hosts in different ecosystems. Highland mouse lemurs, like their congeners in the western dry forests (Greene et al. 2021), harbored abundant *Bifidobacterium* and *Alloprevotella*. Highland sifakas, like their congeners across Madagascar (Greene et al. 2021, 2020; Rudolph et al. 2022; Springer et al. 2017), harbored abundant *Lachnospiraceae* that accounted for nearly 30% of their gut microbiome. And highland brown lemurs, like their conspecifics in western dry forests (Greene et al. 2021), harbored abundant members of the *WCHB1-41* lineage from the *Kiritimatiellae* phylum. The consistency of these patterns highlights deep symbioses between lemur and bacterial genera that were likely established before basal ancestors speciated and radiated across Madagascar (Donohue et al. 2022).

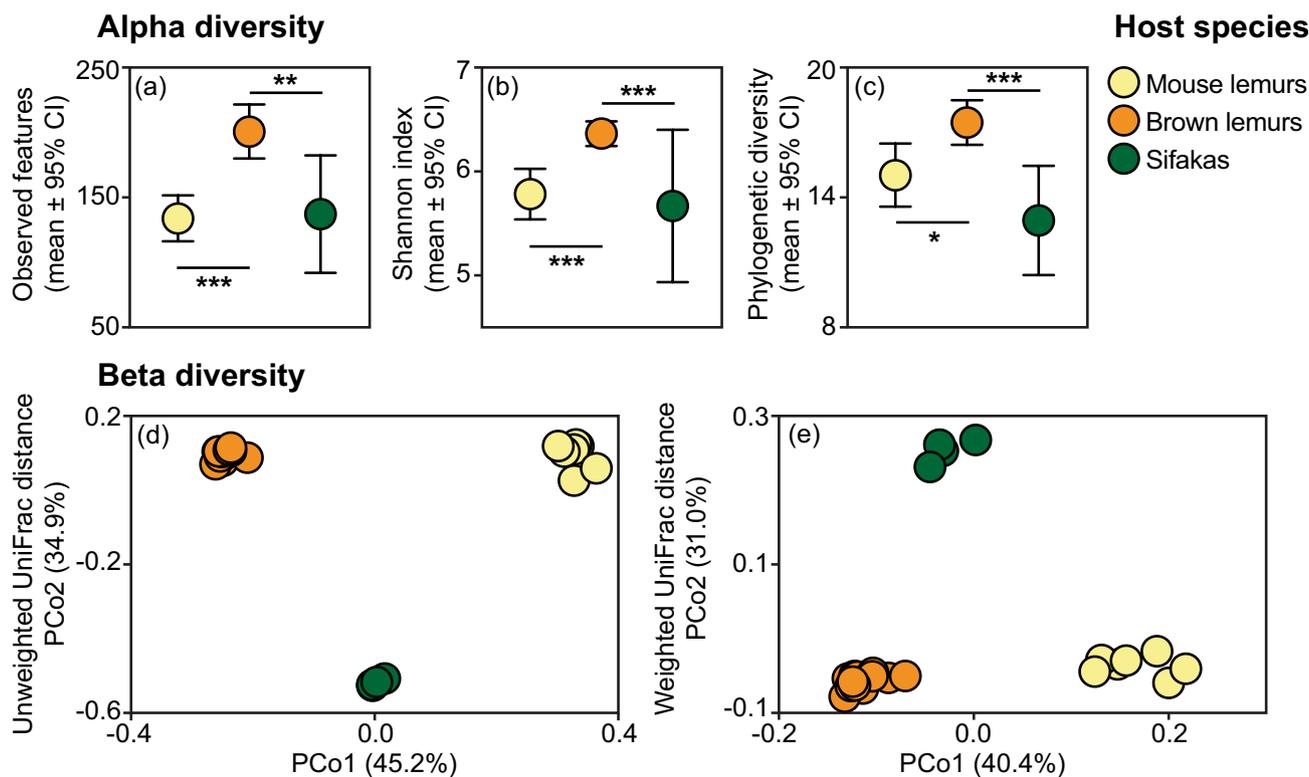


Fig. 3 Microbiome diversity between lemur species inhabiting Madagascar's central highlands, including Goodman's mouse lemurs (yellow), common brown lemurs (orange), and Verreaux's sifakas (green). Depicted here are metrics of alpha diversity, including a

richness, **b** evenness, and **c** phylogenetic breadth, and metrics of beta diversity, including the **d** unweighted and **e** weighted UniFrac metrics. * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$

Our results also add to the conversation about the natural history and species assemblages of the distinct biomes of the highlands (Crowley et al. 2021; Silander et al. 2024; Vorontsova et al. 2016). Once considered human-made landscapes, the highlands are now understood to be complex, dynamic, and heterogeneous mosaics of endemic and anthropogenic features. Perhaps unsurprisingly, the lemurs that persist in these habitats today, including the focal species studied herein, are among the most broadly distributed of their respective genera. This geographic breadth may point to intrinsic ecological flexibility and/or capacity to adapt locally. Although additional study is clearly warranted, we posit that the gut microbiome likely plays a role in supporting nutrition and survival in highland lemurs.

For example, in brown lemurs, the greater diversity of their gut microbiome compared to other lemur species may reflect their use of flexible frugivory (Sato et al. 2016). Although these diversity patterns should be interpreted with caution given our sample sizes, our findings echo those from comparisons of lemur species in other ecosystems showing that a more frugivorous feeding strategy can correspond to a more diverse gut microbiome (Greene et al. 2019; 2020). Consistent with this, two metrics of alpha diversity, namely microbiome evenness and

phylogenetic breadth, were greater among brown lemurs excreting seed-packed vs. leaf-packed fecal samples, a proxy for dietary intakes. This result perhaps suggests that, within species, fruit intake can promote greater gut microbiome diversity. Nevertheless, this result contrasts those from seasonal comparisons of brown lemurs in western Madagascar, where gut microbiome diversity was reduced during the rainy season when fruits and flowers dominate diets (Murillo et al. 2022a). One explanation for this discrepancy relates to nutrition. Emerging evidence points to primate gut microbiomes as being more strongly tuned to macronutrient intake than to plant part (Cui et al. 2022). In the rainy season, perhaps brown lemurs select for less diverse diets of the sweetest fruits and flowers, whereas in the dry season, they fall back on a variety of unripe and fibrous items that promote greater taxonomic and functional microbial diversity. Future studies of brown lemurs in the highlands could beneficially track diets and nutrition across seasons to tease apart determinants of gut microbiome features.

Beyond diversity, we identified specific bacterial genera that varied in abundance in seed-packed vs. leaf-packed feces from brown lemurs. We lack the corresponding feeding and gut-transit time data to link microbial taxa to diet;

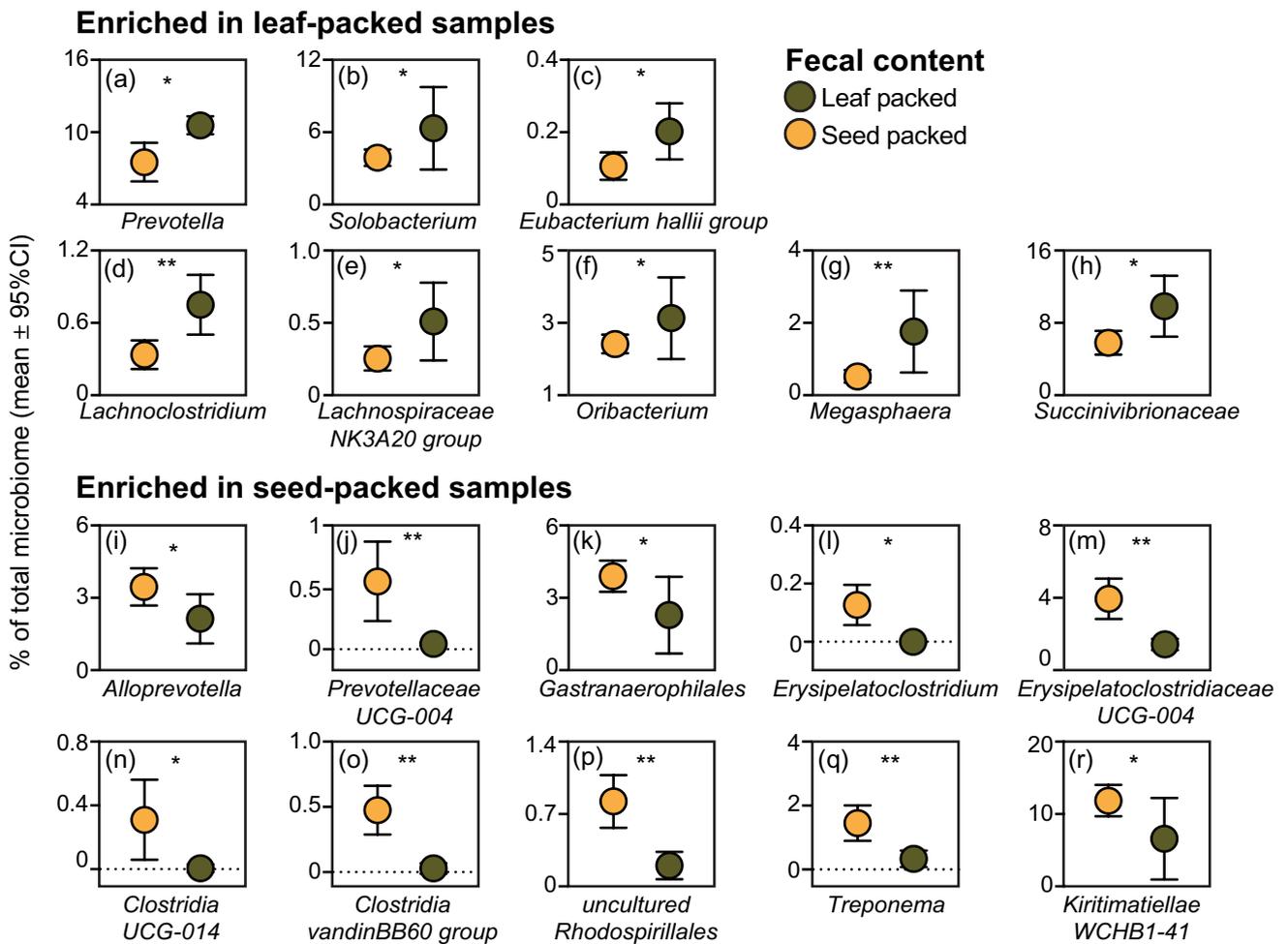
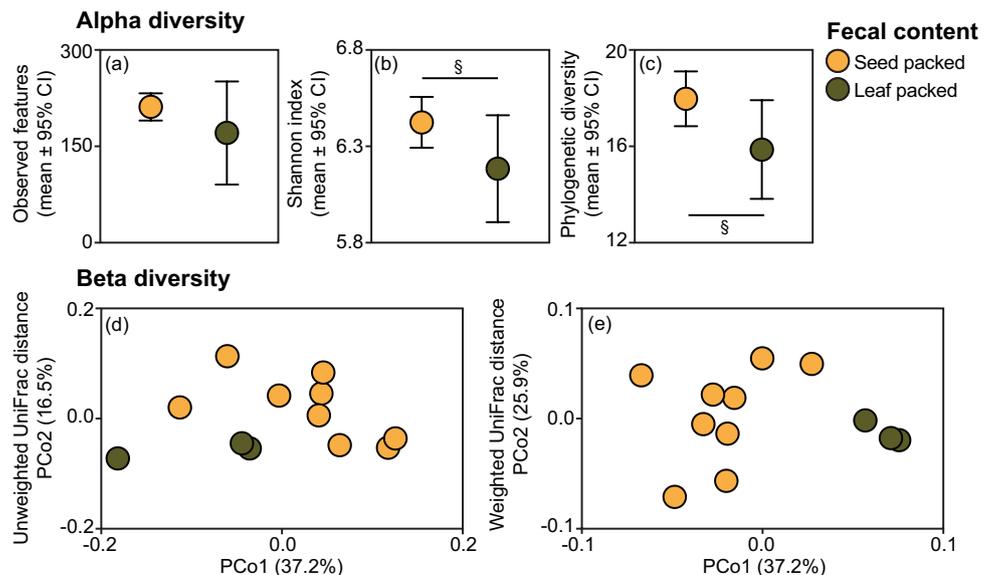


Fig. 4 Microbial taxa that were significantly more abundant in **a–h** leaf-packed (green) vs. **h–r** seed-packed (orange) feces of the common brown lemurs in the Ankafoke protected area. Taxa are resolved to genus-level resolution, except for the case where **h** taxa could not

be assigned below family-level resolution and where **p** taxa include an uncultured genus in an uncultured family of a known order. * $p \leq 0.05$; ** $p \leq 0.01$

Fig. 5 Microbiome diversity between leaf-packed (green) and seed-packed (orange) feces of the common brown lemurs in the Ankafoke protected area. Depicted here are metrics of alpha diversity, including **a** richness, **b** evenness, and **c** phylogenetic breadth, and beta diversity, including **d** unweighted and **e** weighted UniFrac distances. § $p < 0.10$



however, the co-occurrence of fecal content and microbe abundance points to dynamics during transit in the lumen. Differentially enriched taxa included dominant microbes like *Prevotella*, *Solobacterium*, and unassigned *Succinivibrionaceae* that were more abundant in leaf-packed samples. Perhaps these taxa preferentially metabolize leaf fibers or plant defensive compounds or thrive in guts with slower transit time and/or reduced oxygen. In contrast, *WCHB1-41* from the *Kiritimatiellae* family increased nearly twofold in seed-packed samples. This taxon accounted for 6.5% of the microbiome in leaf-packed samples and 11.8% in seed-packed samples; in brown lemurs in western Madagascar, this taxon only accounts for 5.0% of the gut microbiome during the dry season (Greene et al. 2021). In some foraging mammals, *WCHB1-41* and/or *Kiritimatiellae* vary with diets across environments (Guo et al. 2021) and increase when seeds are added to feed (Li et al. 2023). This taxon is implicated in arginine biosynthesis and fatty-acid metabolism, particularly in harsh and high-altitude environments (Guo et al. 2021; Liu et al. 2022). Perhaps *WCHB1-41* helps brown lemurs, especially in the highlands, meet their nutritional demands on diets of unripe and seedy fruits in harsh seasons.

Our results of microbial membership in mouse lemurs and sifakas yield further clues as to the relationship between feeding ecology and primate gut microbiomes. Both mouse lemurs and sifakas in our study had bacterial members that are consistent with the diets of their respective genera. Mouse lemurs uniquely or abundantly harbored microbes like *Bifidobacterium*, *Odoribacter*, and *Alloprevotella* that are implicated in the metabolism of gums (Alarifi et al. 2018; Fu et al. 2019, 2020; Wyatt et al. 1986), as well as *Blautia* and *Peptococcus* that are abundant in insectivore consortia and may metabolize chitin (Delsuc et al. 2014; Teullet et al. 2023). The great abundance of *Lachnospiraceae* in sifaka consortia is likely related to folivory: This *Clostridiales* family within the *Firmicutes* phylum is strongly implicated in plant-fiber metabolism (Biddle et al. 2013) and is routinely found in abundance in diverse folivores and herbivores (Blyton et al. 2019; Rojas et al. 2021).

Our study, though small in scope and largely exploratory, serves as a first step to document the feasibility of gut microbiome research in Madagascar's highlands, with significant potential for follow-up work. We foresee two clear research avenues. First, similar exploratory expeditions could focus on other highland forest sites that are little known and hard to reach, most notably on the western side, like at Mahajebly, Kasijy, and Ambohijanahary to name but a few (Goodman et al. 2018; King et al. 2014; Rakotonirina et al. 2014). This approach could focus particularly on (a) the Crowned and Decken's sifakas that persist in numerous forest patches throughout the west (King et al. 2014; Rakotonirina et al. 2014) and are currently absent from the gut microbiome

literature and (b) the furry-eared dwarf lemur that is found across the highlands and uses seasonal hibernation to combat seasonal resource scarcity (Blanco et al. 2018). The role of the gut microbiome in facilitating primate heterothermy is an area richly deserving of further study (Greene et al. 2022a, b). Second, longitudinal monitoring of Ankafobe's lemurs, and particularly mouse lemurs, could prove clarifying. The mouse lemur population at Ankafobe is stable but small (Blanco et al. 2024a) and experiences high turnover (MBB personal observation). Unlike most sites in the highlands, Ankafobe is logistically easy to reach, requiring only a 4–5-h drive along a good road from the capital city, Antananarivo. Research of Ankafobe's mouse lemurs could track foraging behavior, nutritional quality, torpor expression, and reproduction in individually marked lemurs across seasons (Andriambelason et al. 2021). Such an approach could tease apart determinants of microbiome features that support primate nutrition, metabolism, and resilience in forest fragments and at high elevation.

Ultimately, our study calls for greater inclusion of primates from diverse habitats in gut microbiome science and greater research focus generally on the lemurs of Madagascar's highlands. Whereas habituated populations from well-known study sites with associated long-term monitoring programs offer exceptional opportunity to longitudinally track microbiome structure and function, particularly in well-protected ecosystems (Björk et al. 2019; Murillo et al. 2022b; Springer et al. 2017), less studied lemur species living in a range of habitats offer opportunity to answer different questions. The inclusion of diverse sites in comparisons across Madagascar, particularly when integrated with data on host genetics (Yoder et al. 2016) and focused through a hologenomic lens (Bordenstein and Theis 2015), could shed new light on biogeographic, evolutionary, and ecological processes that shaped the natural history of lemurs and their associated microbiomes.

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Author contributions LKG and MBB conceived and designed the study. LKG, EDF, ADY, and MBB acquired funding. LKG, TA, EDF, AA, TVR, and MBB performed field work with significant logistical facilitation by F Rakotoarison, TR, and F Ratsoavina. Laboratory work was

performed by LKG and MBB in F Ratsoavina and ADY's labs. LKG completed bioinformatic and statistical analysis. LKG and MBB wrote the manuscript. All authors contributed to the final version.

Data availability Raw sequence reads are available online (NCBI SRA PRJNA1186957).

Declarations

Conflict of interest The authors declare no conflict of interest.

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