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HOST MICROBE INTERACTIONS

Gut Site and Gut Morphology Predict Microbiome Structure and Function in Ecologically Diverse Lemurs

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Abstract

Most studies of wildlife gut microbiotas understandably rely on feces to approximate consortia along the gastrointestinal tract. We therefore compared microbiome structure and predicted metagenomic function in stomach, small intestinal, cecal, and colonic samples from 52 lemurs harvested during routine necropsies. The lemurs represent seven genera (*Cheirogaleus*, *Daubentonia*, *Varecia*, *Hapalemur*, *Eulemur*, *Lemur*, *Propithecus*) characterized by diverse feeding ecologies and gut morphologies. In particular, the hosts variably depend on fibrous foodstuffs and show correlative morphological complexity in their large intestines. Across host lineages, microbiome diversity, variability, membership, and function differed between the upper and lower gut, reflecting regional tradeoffs in available nutrients. These patterns related minimally to total gut length but were modulated by fermentation capacity (i.e., the ratio of small to large intestinal length). Irrespective of feeding strategy, host genera with limited fermentation capacity harbored more homogenized microbiome diversity along the gut, whereas those with expanded fermentation capacity harbored cecal and colonic microbiomes with greater diversity and abundant fermentative *Ruminococcaceae* taxa. While highlighting the value of curated sample repositories for retrospective comparisons, our results confirm that the need to survive on fibrous foods, either routinely or in hypervariable environments, can shape the morphological and microbial features of the lower gut.

Keywords Duke Lemur Center · Feeding strategy · Gastrointestinal tract · Gut microbiota · Primate

Introduction

The vertebrate gastrointestinal system serves numerous nutritional functions, including filtering, digesting, absorbing, and eliminating ingested nutrients, toxins, and other compounds [1]. Different segments along the gut vary in their physiological conditions and nutritional roles. We focus predominantly on mammalian hindgut fermenters, i.e., species in which fiber fermentation primarily occurs in the cecum and colon [2]. In these species, food reaches the stomach following preliminary digestion in the mouth, where acidic conditions and muscular contractions continue ingesta breakdown [1]. Sufficiently digested content enters the small intestine, the major site of protein, fat, and carbohydrate processing, where end products of these metabolic processes are readily absorbed [1]. Many plant secondary compounds undergo initial detoxification in the small intestine via conjugation (e.g., glucuronidation) [3]: Conjugates are absorbed for processing in the liver and may re-enter the digestive system via the biliary route for metabolism in the

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large intestine. Lumen digesta is transported by peristaltic action to the cecum and colon: These locations are the powerhouse sites of fiber fermentation, short-chain fatty acid biosynthesis, and absorption in the monogastric gut [1, 4].

The endogenous processes and localized conditions at distinct gastrointestinal sites also select for microbial communities that further mediate digestion [5]. In the stomach, acidic and oxygenated conditions filter environmental inputs and constrain diversity [5–7]. Microbes in the small intestine compete with hosts to scavenge nutrients while contributing to digestion and vitamin biosynthesis [8]. The cecal and colonic microbiotas exhibit a richer array of anaerobic taxa that specialize in recalcitrant fiber fermentation, short-chain fatty acid production, and nutrient recycling and salvage [4, 5, 9]. Fecal microbiomes have been used, to great effect, as proxies for gut consortia. Their study has clarified links between gut microbiomes and host feeding ecology, within the constraints imposed by host phylogeny [10]. However, we lack similar comparative data along the gastrointestinal tracts of diverse hosts to link digestive physiology to microbiome features. Few wildlife studies have compared microbiomes across gut sites, especially in mammals [11–13]. Many of these studies understandably rely on single species (although see 12). Here, we help close this gap by focusing on a large repository of samples from diverse lemurs.

Lemurs, primates from Madagascar, are an excellent, non-traditional system for comparing microbiomes along the gastrointestinal tract linked to digestive physiology. Over 100 species exhibit diverse dietary repertoires, gastrointestinal morphologies, and gut microbiomes [14–17]. Lemur genera that forage primarily on diets high in fats, proteins, sugars, and simple fibers, like aye-ayes (*Daubentonia madagascariensis*) [18], dwarf lemurs (*Cheirogaleus* spp.) [19], and ruffed lemurs (*Varecia* spp.) [20], generally harbor simple gastrointestinal systems with long small intestines; lemur genera that forage on diets containing significant recalcitrant leaf fibers, either year-round or during lean times, like bamboo lemurs (*Hapalemur* spp.) [21], ring-tailed lemurs (*Lemur catta*) [22], brown lemurs (*Eulemur* spp.), and sifakas (*Propithecus* spp.) [23], generally harbor more complex gastrointestinal systems with sacculated ceca and long colons [14, 24–27]. At the extremes, frugivorous dwarf lemurs have a large intestine that is only 15% of their total intestinal length [24], whereas the cecum and colon of seasonally folivorous sifakas comprise over 50% of their total gut length [14]. This tradeoff in small versus large intestinal length highlights that species consuming more bioavailable nutrients rely on absorption in the upper gut; those consuming complex fibers invest in an expanded cecum and colon to maximize retention time, microbial fermentation, and short-chain fatty acid production in the lower gut.

The Duke Lemur Center (DLC), in Durham, NC, has maintained a diverse collection of lemurs under naturalized conditions since 1966. At the end of life, lemurs are humanely euthanized and biological samples are banked during necropsy. This curated repository enabled our team to retrospectively examine microbiome structure and predicted function in the stomach, small intestine, cecum, and colon in species within the *Cheirogaleus*, *Daubentonia*, *Varecia*, *Hapalemur*, *Eulemur*, *Lemur*, and *Propithecus* genera (Fig. 1).

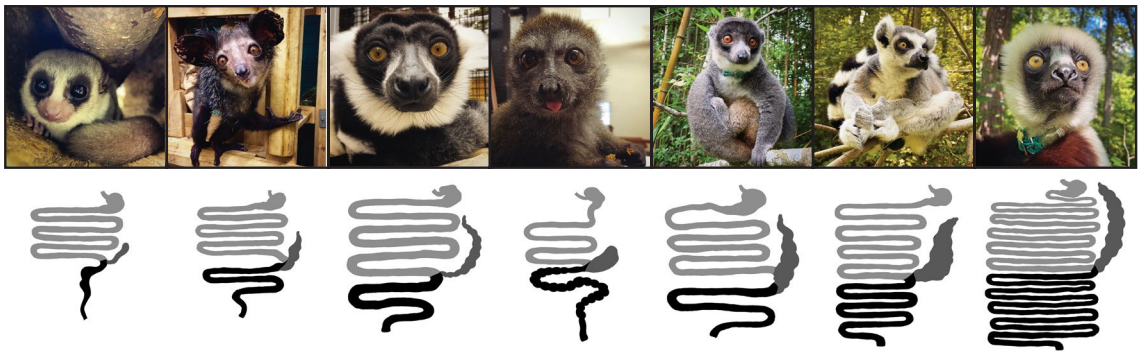
We first determined the microbiome features that consistently define different gut sites across all hosts. We then examined variation within the upper and lower gut microbiomes relative to host traits, including total gut length (relative to body length), morphological investment in fermentation capacity (the ratio of small to large intestinal length), and host phylogenetic affiliation. Under the hypothesis that regional conditions drive microbiome structure, we predicted increasing diversity and decreasing variability in the lower vs. upper gut. Further, we hypothesized that microbiomes are tuned to the digestive processes at each gut site [5] and predicted the greatest fidelity in microbiome features between cecal and colonic communities [27]. Although we expected con-familiar lemurs to harbor some shared microbiome members [15, 28], we nevertheless expected gut length or fermentation capacity to predict aspects of microbiome diversity, variability, composition, and function, especially in the lower gut.

Methods

Subjects and Sampling

The samples stemmed from 52 lemurs, representing seven genera and four families, aseptically collected during necropsies from 2008 to 2017 (Fig. 1). The animals ranged in age from 7 to 32 years. DLC lemurs receive species-specific diets, adjusted per individual and season, that variably comprise chow, produce, insects, and browse. These diets are designed to match, as closely as possible, the seasonal diets foraged by wild lemurs in Madagascar while providing adequate nutrition (supplementary material, Table S1). We selected lemurs whose cause of death was unrelated to the gastrointestinal system, who were not on antibiotics near the time of death, and whose samples were stored at − 80 °C within 3 h of death.

From banked samples that met the above criteria, we targeted the stomach, small intestine, cecum, and distal colon. Where available, we used luminal content. Otherwise, we collected a small slice (< 1 g) from the mid-section of gut segments using sterile instruments while keeping organs frozen. Because the origin of some small-intestine samples was unclear (i.e., duodenum, jejunum, or ileum), we umbrella these sites under the term "small intestine." Using this regimen, we collected 192 samples: individual lemurs



Common name	dwarf lemurs	aye-ayes	ruffed lemurs	bamboo lemurs	brown lemurs	ring-tailed lemurs	sifakas
Genus	*Cheirogaleus*	*Daubentonia*	*Varecia*	*Hapalemur*	*Eulemur*	*Lemur*	*Propithecus*
Family	Cheirogaleidae	Daubentoniidae	Lemuridae	Lemuridae	Lemuridae	Lemuridae	Indriidae
Gut length (gut:body ratio)	long (6.0)[a]	long (6.5)[a,b]	short (4.7)[c]	short (4.1)[c]	short (3.7-4.8)[a,d]	long (5.8)[c]	long (15.5)[c]
Ferm. capacity (SI:CE+CO ratio)	limited (85:15)[a]	limited (64:36)[a,b]	limited (62:38)[c]	expanded (60:40)[c]	expanded (53-59:41-47)[a,d]	expanded (54:46)[c]	expanded (47:53)[c]
# Individuals (ST; SI; CE; CO)	4 (2; 4; 3; 4*)	6 (2; 2; 6; 6)	7 (5; 7; 7*; 7*)	4 (4; 4; 3; 4)	23 (21*; 21; 23; 23)	3 (2; 2; 2; 3)	5 (5; 5; 5; 4)

[a]Campbell, 2003 [24]; [b]Greene & McKenney, 2018 [27]; [c]Campbell *et al.*, 2000 [14]; [d]Schwitzer *et al.*, 2009 [26]
ST = stomach; SI = small intestine; CE = cecum; CO = colon
*one sample removed due to poor quality

Fig. 1 Photographs, gastrointestinal diagrams, and sample sizes of lemur genera featured in the study. Each host genus' common name, scientific name, and phylogenetic family affiliation are provided in the table. Gut morphological features are provided, including gut length (short or long), the ratio of gut length to body length, fermentation capacity (limited or expanded), and the ratio of the small intestines to the cecum and colon. The last row provides the number of individual lemurs in the study, as well as the number of included samples at each gut location per lemur genus. Gut diagrams illustrated by Sally Bornbusch, inspired by previous works [14, 24–26]. Photo of the bamboo lemur provided by Jodi Stirk; other photos by LKG

contributed 3.56 ± 0.7; 2–4 (mean ± SD; range) samples on average. The lower gut was predominately represented by lumen samples (> 95%); the upper gut was represented by a mixture of lumen (34%) and mucosal samples (66%).

Sequencing and Bioinformatics

We extracted gDNA using Qiagen's DNeasy PowerSoil Kit. We followed the suggested workflow but reduced starting volumes to ~ 0.1 g and heated samples at 60 °C for 10 min prior to bead beating. We shipped aliquots to the Primate Microbiome Project (Nebraska Food for Health Center, Lincoln, NE) for amplicon sequencing. We targeted the V3–V4 region of the 16S rRNA gene using the 341F and 805R primers, 2 × 300 paired-end reads, and Illumina's MiSeq platform. We processed reads in QIIME2 (version 2019.10) [29]. Sequences were filtered for low-quality, chimeric, and singleton reads. Five samples sequenced poorly; all other samples were represented by > 4000 high-quality reads per sample. We binned reads into Amplicon Sequence Variants (ASVs) based on 100% sequence similarity. We assigned ASV taxonomy using QIIME2's feature-classifier plugin with the Silva 132 99% Naïve Bayes classifier. We removed chloroplast and mitochondrial sequences. We also removed four outlier samples that contained an over-representation of one taxon, including one stomach sample from an *E. rubriventer* comprising > 80% chloroplast sequences (identified prior to filtering); the cecum and colon samples from one *V. variegata* comprising 30–50% *Escherichia coli* sequences; and one mucosal colon sample from a *C. medius* comprising > 70% *Campylobacter* sequences. Our final dataset contained 183 samples, of which 40, 46, 48, and 49, respectively, derived from the stomach, small intestine, cecum, and colon.

We computed alpha diversity, using community richness (observed ASVs) and evenness (the Shannon index), and beta diversity, using unweighted and weighted UniFrac distances [30]. Because the two UniFrac metrics yielded largely identical results in downstream statistical analyses, we report only those of the unweighted metric. We ran sequences through PICRUSt2, which predicts the presence and abundance of metabolic pathways per sample from microbial identification [31].

Statistical Analyses of Microbiome Diversity

Because the strength of our dataset lies in comparisons across gastrointestinal sites and lemurs, and because any individual may have been in dysbiosis at the time of death, we focused our statistical approach by averaging values across congeners.

We focus on "gut sites" (i.e., stomach, small intestine, cecum, colon) or "gut regions" (i.e., the upper or lower gut). We use three categorical variables to characterize host traits. Lemur genera (1) belong to the Lemuridae or a non-Lemuridae family; (2) have short or long gastrointestinal systems based on a cutoff of gut length > 5 × body length; and (3) have expanded versus limited fermentation capacity in their lower gut, based on a cutoff of cecum + colon length ≥ 40% of total gut length (Fig. 1).

Alpha diversity metrics were normally distributed. We ran two analyses of variance (ANOVA) in RStudio (version 1.3.959) [32] with R software (version 4.0.2) [33], using richness or evenness as the dependent variable and gut site as the independent variable. We ran two additional ANOVAs in which we retained alpha diversity as the dependent variable but entered gut region interacted with fermentation capacity and gut length as the dependent variables. We used Tukey's post hoc tests to determine pairwise comparisons.

To assess beta diversity, we computed permutational analysis of variance using distance (adonis) with the vegan package (version 2.5.7) [34]. We used unweighted UniFrac distances as the dependent variable and gut site as the independent variable. We used the pairwiseAdonis package (version 0.0.1) for post hoc comparisons between sites [35]. We ran an additional PERMANOVA in which we retained unweighted UniFrac scores as the dependent variable, but nested taxonomic affiliation, gut length, and fermentation capacity within gut region. We used the ape package (version 5.5) to calculate Principal Coordinates (PCo) of unweighted UniFrac distances [36]. We retained the top three PCos and used them as the dependent variables in ANOVAs that included gut region, taxonomic affiliation, gut length, and fermentation capacity as independent variables.

We determined which gut sites harbored the most similar microbiomes within individuals by retaining unweighted UniFrac values that compared two different sites derived from the same lemur. We computed Kruskal–Wallis tests with Dunn's multiple comparisons in GraphPad Prism (version 9.1.2), for which we entered comparisons between the stomach vs. small intestinal microbiomes, cecal vs. colonic microbiomes, and all upper vs. all lower gut microbiomes.

Statistical Analyses of Microbiome Composition and Predicted Function

To compare composition across gut regions and hosts, we collapsed our ASV table across congeneric hosts per gut site and at microbial genus-level resolution. Regarding metagenomic function, we averaged the relative abundances of metabolic pathways across congeners per gut site. We used linear discriminant analysis effect size (LEfSe) to determine which microbial genera or metagenomic pathways were significantly enriched in the upper or lower gut by entering data from each gut site per host genus [37]. We included host genus as the "subject" variable. Because LEfSe cannot account for multiple variables concurrently, we next analyzed patterns separately within the upper and lower gut. We compared profiles between the stomach and small intestinal microbiomes and between the cecal and colonic microbiomes. Next, we compared patterns between host genera with expanded vs. limited fermentation capacity. We applied the Benjamini–Hochberg correction factor across all analyses to account for multiple comparisons [38].

Results

Microbiome Diversity Along the Gastrointestinal Tract

The lemurs' upper gut microbiomes were less diverse than were their lower gut microbiomes (Fig. 2). We found a significant effect of gut site on richness (ANOVA: $F_{3,24} = 12.04$, $p < 0.001$, Fig. 2a) and evenness (ANOVA: $F_{3,24} = 8.27$, $p < 0.001$, Fig. 2b). Post hoc tests clarified that the stomach and small intestinal microbiomes were similarly diverse (richness, $p = 0.255$; evenness, $p = 0.321$), as were the cecal and colonic microbiomes ($p > 0.997$ for both metrics). The overall effect of gut site was driven by differences in diversity between the upper and lower gut microbiomes. The stomach microbiome was less rich than either the cecal or colonic microbiome ($p < 0.031$ for both comparisons) and trended towards being less even ($p < 0.1$ for both comparisons). The small intestinal microbiome was less diverse than either the cecal or colonic microbiome (richness: $p < 0.001$ for both comparisons; evenness: $p < 0.002$ for both comparisons).

The upper and lower gut microbiomes across lemur genera were structurally distinct. We found an effect of gut site on unweighted UniFrac distances (PERMANOVA: $F_{3,24} = 5.26$, $R_2 = 0.40$, $p < 0.001$; Fig. 2c), with gut site explaining 40% of the variance. Post hoc tests revealed no difference between the stomach and small intestinal microbiomes ($p = 0.906$) or the cecal and colonic microbiomes ($p = 1.0$). The stomach microbiome differed from both the cecal and colonic microbiomes ($p = 0.018$, for both comparisons), and the small intestinal microbiome differed from both the cecal ($p = 0.012$) and colonic ($p = 0.006$) microbiomes.

Within individual lemurs, we found significant differences between pairwise unweighted UniFrac comparisons across gut sites (Kruskal–Wallis: $H = 71.57$, $p < 0.001$; Fig. 2d). Post hoc tests clarified that cecal and colonic microbiomes harbored the most similar microbiomes overall: the differences between these microbiomes were significantly smaller than were the differences between the stomach and small intestinal microbiomes and between all upper and lower gut sites ($p < 0.001$ for both comparisons).

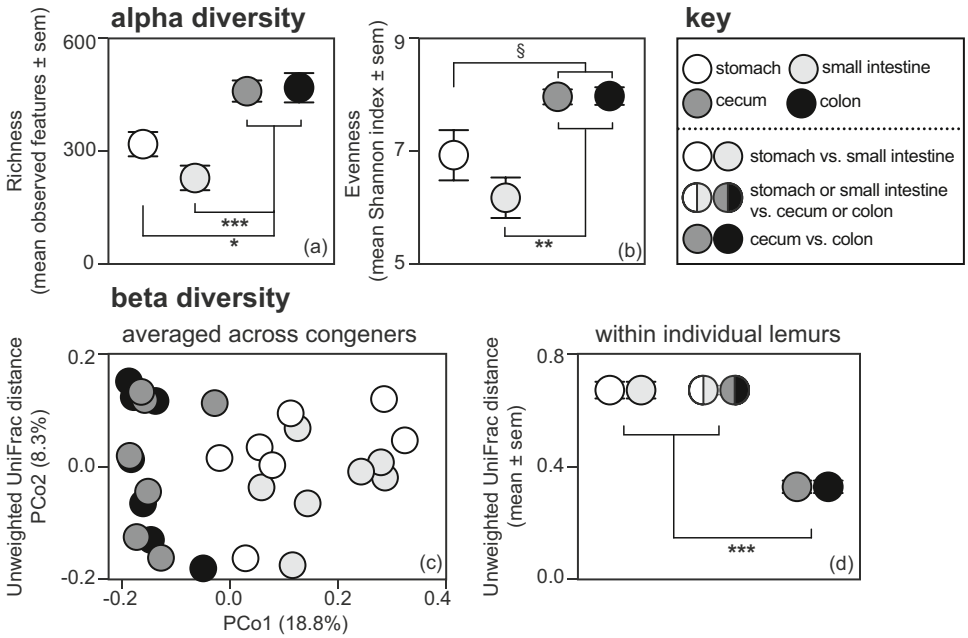


Fig. 2 Diversity in the microbiome at four gastrointestinal sites across seven lemur genera, including the stomach (white), small intestine (silver), cecum (grey), and colon (black). Depicted are measures of alpha diversity, including microbiome (**a**) richness as captured by Observed Features and (**b**) evenness as captured by the Shannon index, and of beta diversity, including unweighted UniFrac distances graphed (**c**) in Principal Coordinate space averaged across congeners and (**d**) as pairwise comparisons within individual lemurs across gut sites. *$p<0.05$; **$p<0.01$; ***$p<0.001$; §$p<0.1$

Microbiome Composition and Predicted Function Along the Gastrointestinal Tract

The microbiomes were generally dominated by *Firmicutes*, *Bacteroidetes*, *Proteobacteria*, *Actinobacteria*, and *Epsilonbacteraeota.* We detected considerable variation within and between host genera and gut sites; however, the cecal and colonic microbiomes were the most similar (supplementary material, figure S1). LEfSe identified 43 microbes that were significantly enriched in the lemurs' upper ($n=15$) or lower ($n=28$) gut microbiomes (Fig. 3). For example, the upper gut contained greater abundances of *Sarcina* (log(LDA) = 4.89, $p=0.017$) and *Bifidobacterium* (log(LDA) = 3.13, $p=0.039$). In contrast, the lower gut was dominated by archaeal methanogens from *Methanomethylophilaceae* (log(LDA) = 2.71, $p=0.044$) and the bacterial taxa *Bacteroides* (log(LDA) = 4.32, $p=0.048$) and *Treponema2* (log(LDA) = 3.84, $p=0.045$), along with genera from the *Lachnospiraceae*, *Ruminococcaceae*, and *Erysipelotrichaceae* families.

Predicted metagenomic function also varied along the gastrointestinal tract: we identified 81 metabolic pathways that differed between the upper and lower gut, including tradeoffs in amino acid cycling, fermentation, plant secondary compound metabolism, and vitamin biosynthesis (Fig. 4). The upper gut microbiome showed greater capacity for amino acid degradation (Fig. 4a), including of histidine, arginine, and ornithine (log(LDA) > 2.10, $p<0.012$ for all comparisons); the lower gut showed greater capacity for amino acid biosynthesis (Fig. 4b), including of lysine, tryptophan, isoleucine, and threonine (log(LDA) > 2.50, $p<0.04$ for all comparisons). The upper gut microbiome showed greater capacity for vitamin B_{12} biosynthesis via aerobic pathways (log(LDA) = 2.17, $p=0.002$; Fig. 4c) and cofactor Q biosynthesis (log(LDA) = 2.47, $p=0.005$), whereas the lower gut microbiome had greater capacity for the final stages of B_{12} biosynthesis (log(LDA) = 2.62, $p=0.04$; Fig. 4d) and cofactor A biosynthesis (log(LDA) = 2.29, $p=0.038$). The upper gut microbiome showed greater capacity for metabolizing protocatechuate (log(LDA) = 2.09, $p=0.005$; Fig. 4e), whereas the lower gut microbiome was enriched for pathways related to galacturonic acid metabolism (log(LDA) = 2.65, $p=0.007$; Fig. 5f) and pyruvate fermentation to acetate and lactate (log(LDA) = 2.45, $p=0.04$; Fig. 4g) and propanoate (log(LDA) = 2.68, $p=0.05$; Fig. 4g). We also detected more abundant pathways related to the TCA and glyoxylate cycles in the upper gut microbiomes.

Gut Morphology and the Gut Microbiome

We found that fermentation capacity, more than total gut length, influenced the lemurs' microbiomes (Fig. 5). Regarding alpha diversity, we found no main effect of gut length (ANOVAs: richness, $F_{1,22}=0.017$, $p=0.898$; evenness, $F_{1,22}=0.097$, $p=0.759$) or fermentation capacity (ANOVAs: richness, $F_{1,22}=0.292$, $p=0.594$; evenness, $F_{1,22}=0.630$, $p=0.436$) and no significant interaction between gut region and gut length (ANOVA: richness, $F_{1,22}=2.280$, $p=0.145$; evenness, $F_{1,22}=0.960$, $p=0.338$). However, we detected a significant interaction between gut region and fermentation capacity (ANOVAs: richness, $F_{1,22}=12.319$, $p=0.002$; evenness, $F_{1,22}=8.020$, $p=0.01$; Fig. 5a,b). Post hoc tests revealed differences between the upper and lower gut microbiomes for hosts with expanded fermentation capacity ($p<0.001$ for both metrics) but not with limited fermentation capacity (richness: $p=0.367$; evenness: $p=0.656$).

Fig. 3 Microbial taxa significantly enriched in the upper (stomach and small intestinal) or lower (cecal and colonic) gut microbiomes across seven lemur genera, including *Cheirogaleus* (C), *Daubentonia* (D), *Varecia* (V), *Hapelemur* (H), *Eulemur* (E), *Lemur* (L), and *Propithecus* (P). The heat map shows the relative abundance of microbes (% of total microbiome) in the microbiome across congeners at each gut site, with rows depicting microbial genera and columns depicting host genera. Microbial taxonomy is shown to the right of each row, when possible, to genus level. "Unassigned" refers to the summation of all sequences that could be taxonomically assigned below the lowest resolution presented

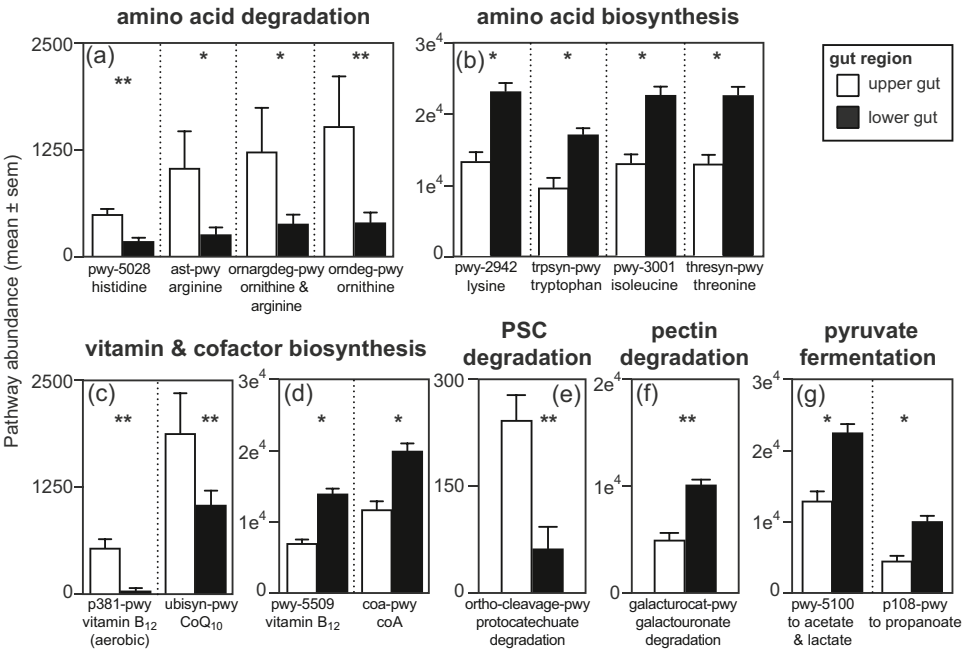


Fig. 4 Predicted metagenomic function in the microbiome across seven lemur genera in the upper (white) and lower (black) gut. Bars indicate the relative abundance of metabolic pathways related to (**a**) amino acid degradation, (**b**) amino acid biosynthesis, (**c**, **d**) vitamin and cofactor biosynthesis, (**e**) plant secondary compound (PSC) metabolism, (**f**) pectin degradation, and (**g**) pyruvate fermentation. Pathway names and numbers are from the MetaCyc database. $*p < 0.05$; $**p < 0.01$

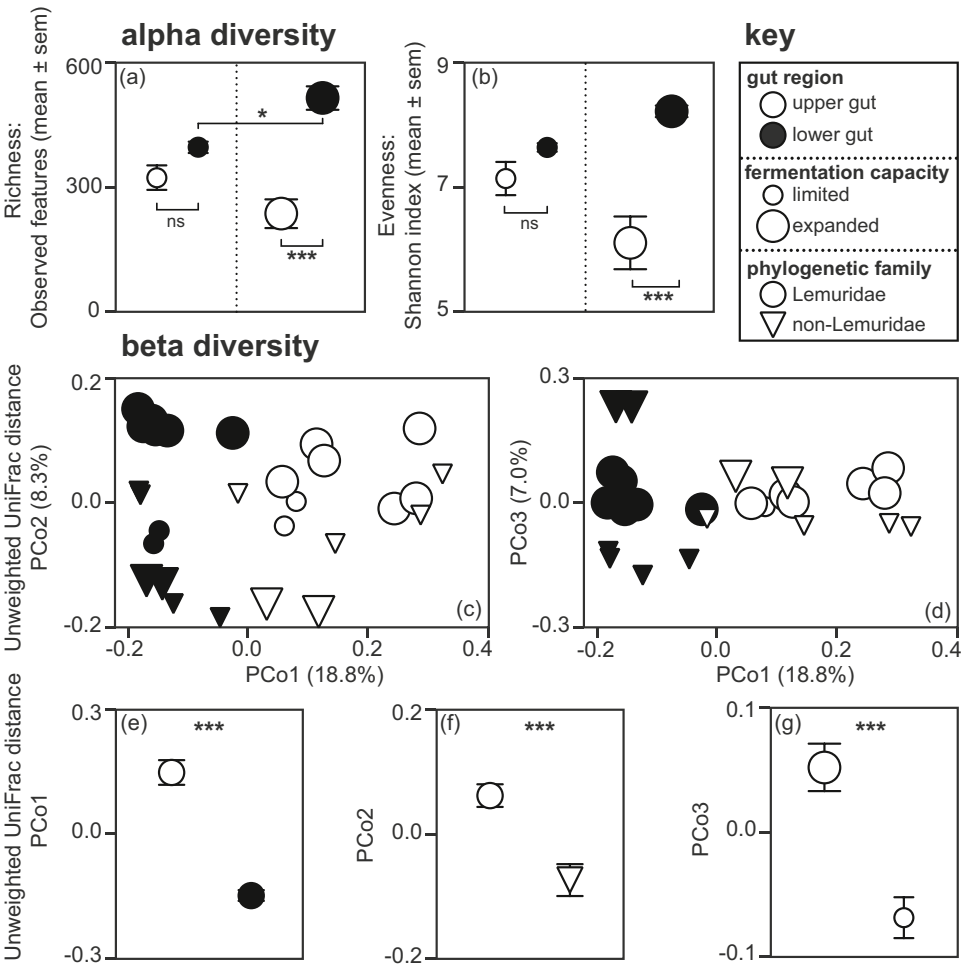


Fig. 5 Diversity in the microbiomes across seven genera of lemurs relative to gut region, fermentation capacity, and phylogenetic family affiliation. Depicted here are measures of alpha diversity, including microbiome (**a**) richness (Observed Features) and (**b**) evenness (Shannon index) in the upper (white) and lower (black) gut microbiomes of lemur genera with limited (small size) or expanded (large size) fermentation capacity. Beta diversity, as captured by unweighted UniFrac distances, is graphed in (**c**, **d**) Principal Coordinate space relative to gut site and fermentation capacity, and relative to whether hosts belong to the Lemuridae family (circles) or not (triangles). **e** PCo1 is further graphed relative to gut region; **f** PCo2 is graphed relative to the hosts' phylogenetic affiliation; and **g** PCo3 is graphed relative to the hosts' fermentation capacity. $*p < 0.05$; $***p < 0.001$; ns $p > 0.10$

Hosts with expanded versus limited fermentation capacity had richer consortia in their lower guts ($p = 0.042$), though they had comparable evenness ($p = 0.488$).

Regarding beta diversity, we found taxonomic affiliation, nested within gut region, predicted unweighted UniFrac distances (PERMANOVA: $F_{3,20} = 12.50$, $R_2 = 0.533$, $p < 0.001$; Fig. 5c,d). While accounting for this variation, we found a modest effect of total gut length nested within gut region ($F_{2,20} = 1.838$, $R_2 = 0.052$, $p = 0.076$), which explained 5% of the variance in our dataset; but a significant effect of fermentation capacity nested within gut region ($F_{2,20} = 4.557$, $R_2 = 0.130$, $p = 0.001$), which explained 13% of the variance in our dataset. Of the top PCos calculated from unweighted UniFrac distances, PCo1 was influenced by gut region (ANOVA: $F_{1,23} = 77.379$, $p < 0.001$; Fig. 5e), PCo2 was influenced by taxonomic affiliation (ANOVA: $F_{1,23} = 18.353$, $p < 0.001$; Fig. 5f), and PCo3 was influenced by fermentation capacity (ANOVA: $F_{1,23} = 19.451$, $p < 0.001$; Fig. 5g). We detected no other significant relationships between the top PCos and host traits.

When examining the lemurs' upper and lower gut microbiomes separately, we found no single taxon or metabolic pathway that was enriched in stomach vs. small intestinal microbiomes or in cecal vs. colonic microbiomes. However, when computing analyses relative to fermentation capacity, we found 23 taxa and 24 metabolic pathways that were differentially enriched in the hosts' lower gut microbiomes, representing tradeoffs predominately in *Lachnospiraceae* and *Ruminococcaceae* taxa (Fig. 6) and in pathways linked to sugar metabolism (supplementary material, figure S2).

In the lower gut, hosts with expanded fermentation capacity had microbiomes enriched for the *R7 group* from the *Christensenellaceae* family and *UCG-005*, *UCG-010*, *Ruminoclostridium5*, and the *NK4A214 group* from the *Ruminococcaceae* family ($\log(\text{LDA}) > 3.50$, $p < 0.05$ for all comparisons), for example. Hosts with limited fermentation capacity had microbiomes enriched for *Lachnoclostridium* and *Anaerostipes* from the *Lachnospiraceae* family ($\log(\text{LDA}) > 3.30$, $p < 0.05$ for both comparisons), for example. Hosts with limited fermentation capacity had greater capacity for sugar degradation (figure S2a), including of fucose and rhamnose, glucose, and hexitol ($\log(\text{LDA}) > 2.20$, $p < 0.05$ for all comparisons), and greater capacity for vitamin K1 and K2 biosynthesis ($\log(\text{LDA}) > 2.20$, $p < 0.05$ for both pathways; figure S2b). In contrast, hosts with expanded fermentation capacity had greater metagenomic capacity for pyruvate fermentation ($\log(\text{LDA}) = 2.65$, $p = 0.048$; figure S2c).

Discussion

We used a curated sample repository to characterize the stomach, small intestinal, cecal, and colonic microbiomes of diverse lemurs while illuminating how variation in gut morphology can underlie microbiome features. In general, microbial richness and evenness increased along the gastrointestinal tract, while variability decreased. Microbiome membership and function differed between the upper and lower gut, reflecting regional tradeoffs in conditions and macronutrients [5]. These patterns, particularly those in the cecum and colon, were modulated by the hosts' fermentation capacity, as measured by the ratio of small to large intestines. Lemur genera with expanded fermentation capacity harbored greater microbiome diversity and enrichment for *Ruminococcaceae* in their lower guts [39]. In contrast, hosts with more limited fermentation capacity harbored more homogenized microbiome diversity across gut sites and enriched capacity for sugar metabolism in their lower guts. Lemurs that eat more digestible diets versus lemurs that must sometimes rely on more fibrous items share gut morphological and microbiome features, irrespective of feeding strategy. We suggest that the digestibility of staple and fallback foods, more so than food type, can shape the evolution of host-microbial symbioses in the gut.

Across study lemurs, we found distinct microbiome communities at each gut site, which makes sense considering the different digestive processes and physiological conditions at each gut site [1]. In the stomach and small intestine, digestion occurs under acidic and oxygenated conditions [1]. The microbiomes at these sites showed corresponding enrichment for acid- or oxygen-tolerant microbes, including *Sarcina* [40], *Proteobacteria* [41], and in the case of the brown lemurs, *Helicobacter* [42]. We likewise found greater abundances of *Clostridium* and various *Lactobacillales* that are known to inhabit mammalian upper guts [5, 7, 43]. In addition, there was greater metagenomic capacity to degrade amino acids in the upper gut, potentially linked to ingested protein, and capacity to degrade protocatechuate, an intermediate compound produced during the microbial metabolism of aromatics [44].

The lemurs' lower gut microbiomes were richer and more evenly distributed and comprised anaerobic taxa known to ferment fiber, like genera within the *Ruminococcaceae* and *Lachnospiraceae* families [39] and methanogenic archaea [45]. Relative to the upper gut, we observed greater homogenization between cecal and colonic microbiomes within individuals, across congeners, and distant relatives. Lemur feces thus provide a decent proxy for cecal and colonic microbiomes, but do not provide good representations of stomach and small intestinal consortia. Regarding functionality, the lemurs' lower gut microbiomes showed greater capacity for amino acid biosynthesis, pectin metabolism, and pyruvate fermentation, which highlight microbial roles in nutrient recycling, fiber degradation, and short-chain fatty acid production [9]. These patterns support the hypothesis that localized conditions and digestive processes at different gut sites select for specific microbiotas [5].

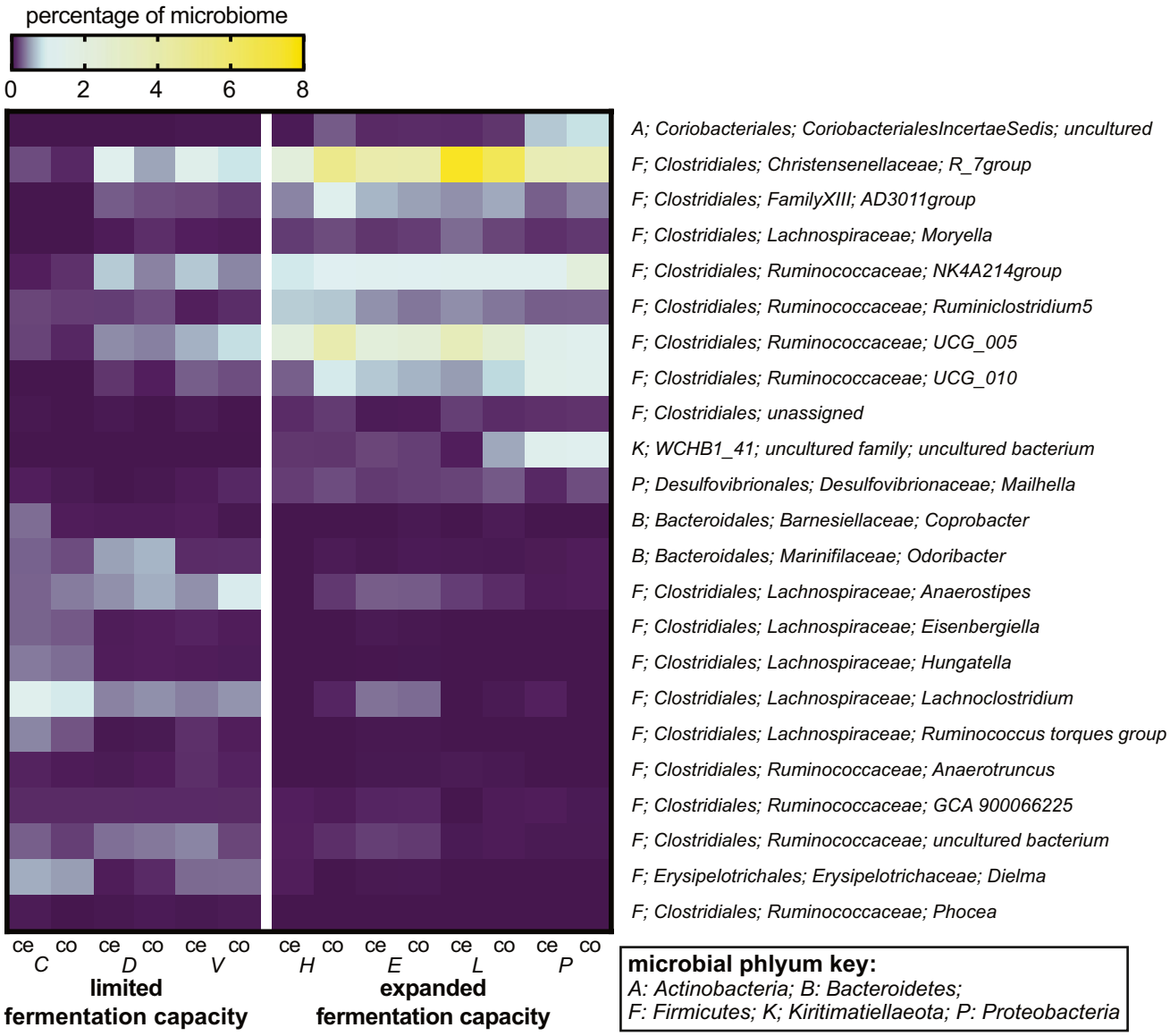


Fig. 6 Microbial taxa significantly enriched in the cecal (ce) and colonic (co) microbiomes of host lemurs with limited and expanded fermentation capacity, including *Cheirogaleus* (C), *Daubentonia* (D), *Varecia* (V), *Hapelemur* (H), *Eulemur* (E), *Lemur* (L), and *Propithecus* (P). The heat map shows the relative abundance of microbes (% of total microbiome) in the microbiome across congeners at each lower gut site, with rows depicting microbial genera and columns depicting host genera relative to fermentation capacity. Microbial taxonomy is shown to the right of each row, when possible, to genus level. "Unassigned" refers to the summation of all sequences that could be taxonomically assigned below the lowest resolution presented

Our results further suggest that variation in the lemurs' lower gut microbiomes is shaped by the morphological capacity for fermentation. Specifically, host genera with expanded fermentation capacity in the lower gut had correspondingly more diverse and compositionally distinct microbiomes in the cecum and colon. In particular, expanded fermentation capacity correlated to enrichment for *Christensenellaceae R7* and *Ruminococcaceae* taxa, whereas limited fermentation capacity was associated with *Lachnospiraceae* taxa. Both *Ruminococcaceae* and *Lachnospiraceae* have genetic potential to ferment complex carbohydrates and substrates, with the former more tuned to cellulose, hemicellulose, and xylan and the latter more tuned to starch, pectin, and chitin [39]. *Ruminococcaceae* taxa, in particular, have been established as critical fermenters that contribute to short-chain fatty acid production [46]. The *Christensenellaceae R7* and *Ruminococcaceae UCG-005* genera may emerge as specific markers for leaf-fiber fermentation in lemurs [47]. Outside of lemurs, these taxa are gaining recognition for their cellulolytic and fermentative capacity [48] and their positive

association with human health [49]. That the relative length of the lemurs' lower guts, but not their entire gastrointestinal tracts, predicted microbiome features echoes previous work on gut morphology and dietary ecology in various primates [16, 25] and highlights the significance of fiber digestion in shaping primate evolution [17].

Our study was possible because of the DLC's effort to swiftly collect, curate, and comparably bank biological samples upon necropsy. Though this dataset presents a rare opportunity to study digestive physiology and gut microbiomes using humane approaches, it comes with limitations. Our study relies on geriatric and sick animals in captivity: some findings may be biased by lemur age, condition, and human management. We collapsed samples from mucosal and lumen sources to boost power, understanding that these habitats select for different microbes: The reduced diversity of upper gut consortia could be reflected in the greater inclusion of mucosal samples [50]. We further collapsed samples from multiple points along the small intestine because the specific location of origin was often unclear. We found the greatest microbiome variation within and between stomach, and especially, small intestinal samples. Future studies could specifically examine the duodenal, jejunoileal, and ileal microbiomes in lemurs and clarify differences between mucosal and lumen consortia [5, 43, 50]. While we strongly caution against the invasive research to overcome such limitations in wildlife, we recommend accredited facilities curate gut content for future retrospective studies.

The inclusion of additional methodological approaches could also strengthen our results, especially shotgun metagenomic sequencing and short-chain fatty acid profiling. Here, we used predicted metagenomic function, which is cost-effective but inherently relies on microbial identity to assume microbial function. This approach thus excludes microbes with uncertain classifications and those unknown to the software from host species, like lemurs, that are underrepresented in online microbial databases. Future work to assay the concentrations of short-chain fatty acid in the lemurs' samples or culture their consortia under different conditions could establish causal links between microbial identity and function relative to host fermentation capacity. Consideration of morphological traits beyond gut length, such as surface area, volume, sacculation, and retention time, though hard to find in the literature, could also prove beneficial.

Although our study subjects lived in captivity, they are representatives of their wild kin; our results shed light on the mechanisms that underlie variation in gut microbiotas. Evolution in the diverse, hypervariable, and stochastic environments that characterize Madagascar required lemurs to withstand food scarcity [51]. Species strongly reliant on easily digestible foodstuffs, like fruits and grubs, are either restricted to the more plenteous rainforests (*Varecia*), sustain hibernation during the dry season (*Cheirogaleus*) [52], or evolved morphological toolkits to extract structurally defended items (*Daubentonia*) [18]. Absent such strategies, lemurs routinely or periodically rely on microbial fermentation of fibrous foods in the hindgut [15, 47, 53]. Despite their disparate feeding strategies—ranging from frugivory to folivory—these species host rich and diverse microbiomes in their cecum and colon comprising the fermentative taxa that enable fiber digestion. Our results highlight that the need to survive on seasonal or emergency fallback foods may shape morphology [16, 17] and microbiotas in the lower gut across lemurs and ultimately may shape species diversity, adaptation, and resilience.

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Author Contribution LKG and EAM conceived and designed the study with help from EEE. EEE curated the sample repository. CW and EAM collected samples, LKG performed sample extraction. WG, SH, and JBC performed sequencing and bioinformatic analyses. LKG completed statistical analyses and data visualizations. LKG and EAM drafted the manuscript; all authors contributed to the final version.

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Data Availability Sequences and metadata are available upon reasonable request from the lead or senior authors.

Declarations

Competing Interests The authors declare no competing interests.

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References

1. Stevens CE, Hume ID (2004) Comparative physiology of the vertebrate digestive system, 2nd edn. Cambridge University Press, Cambridge
2. Hume ID (1997) Fermentation in the hindgut of mammals. In: Mackie RI, White BA (eds) Gastrointestinal Microbiology, vol 1. Gastrointestinal Ecosystems and Fermentations. Chapman & Hall, International Thomson Publishing, pp 84–115

3. Manach C, Scalbert A, Morand C, Rémésy C, Jiménez L (2004) Polyphenols: food sources and bioavailability. Am J Clin Nutr 79:727–747. https://doi.org/10.1093/ajcn/79.5.727
4. Stevens CE, Hume ID (1998) Contributions of microbes in vertebrate gastrointestinal tract to production and conservation of nutrients. Physiol Rev 78:393–427. https://doi.org/10.1152/physrev.1998.78.2.393
5. Martinez-Guryn K, Leone V, Chang EB (2019) Regional diversity of the gastrointestinal microbiome. Cell Host Microbe 26:314–324. https://doi.org/10.1016/j.chom.2019.08.011
6. Beasley DE, Koltz AM, Lambert JE, Fierer N, Dunn RR (2015) The evolution of stomach acidity and its relevance to the human microbiome. PLoS ONE 10:e0134116. https://doi.org/10.1371/journal.pone.0134116
7. Lee W, Hayakawa T, Kurihara Y, Hanzawa M, Sawada A, Kaneko A et al (2021) Stomach and colonic microbiome of wild Japanese macaques. Am J Primatol 83:e23242. https://doi.org/10.1002/ajp.23242
8. Kastl AJ Jr, Terry NA, Wu GD, Albenberg LG (2020) The structure and function of the human small intestinal microbiota: current understanding and future directions. Cell Mol Gastroenterol Hepatol 9:33–45. https://doi.org/10.1016/j.jcmgh.2019.07.006
9. Dearing MD, Kohl KD (2017) Beyond fermentation: other important services provided to endothermic herbivores by their gut microbiota. Int Comp BioL 57:723–731. https://doi.org/10.1093/icb/icx020
10. Rojas CA, Ramirez-Barahona S, Holekamp KE, Theis KR (2021) Host phylogeny and host ecology structure the mammalian gut microbiota at different taxonomic scales. Anim Microbiome 3:1–18. https://doi.org/10.1186/s42523-021-00094-4
11. Gillman SJ, McKenney EA, Lafferty DJ (2020) Wild black bears harbor simple gut microbial communities with little difference between the jejunum and colon. Sci Rep 10:20779. https://doi.org/10.1038/s41598-020-77282-w
12. Ingala MR, Simmons NB, Wultsch C, Krampis K, Speer KA, Perkins SL (2018) Comparing microbiome sampling methods in a wild mammal: fecal and intestinal samples record different signals of host ecology, evolution. Front Microbiol 9:803. https://doi.org/10.3389/fmicb.2018.00803
13. Clayton JB, Shields-Cutler RR, Hoops SL, Al-Ghalith GA, Sha JC, Johnson TJ et al (2019) Bacterial community structure and function distinguish gut sites in captive red-shanked doucs (*Pygathrix nemaeus*). Am J Primatol 81:e22977. https://doi.org/10.1002/ajp.22977
14. Campbell JL, Eisemann JH, Williams CV, Glenn KM (2000) Description of the gastrointestinal tract of five lemur species: *Propithecus tattersalli*, *Propithecus verreauxi coquereli*, *Varecia variegata*, *Hapalemur griseus*, and *Lemur catta*. Am J Primatol 52:133–142. https://doi.org/10.1002/1098-2345(200011)52:3%3c133::AID-AJP2%3e3.0.CO;2-%23
15. Greene LK, Williams CV, Junge RE, Mahefarisoa KL, Rajaonarivelo T, Rakotondrainibe H et al (2020) A role for gut microbiota in host niche differentiation. ISME J 14:1675–1687. https://doi.org/10.1038/s41396-020-0640-4
16. Chivers DJ, Hladik CM (1980) Morphology of the gastrointestinal tract in primates: comparisons with other mammals in relation to diet. J Morphol 166:337–386. https://doi.org/10.1002/jmor.1051660306
17. Lambert JE (1998) Primate digestion: interactions among anatomy, physiology, and feeding ecology. Evol Anthropol 7:8–20. https://doi.org/10.1002/(SICI)1520-6505(1998)7:1%3c8::AID-EVAN3%3e3.0.CO;2-C
18. Sterling EJ (1994) Aye-ayes: specialists on structurally defended resources. Folia Primatol 62:142–154. https://doi.org/10.1159/000156771
19. Fietz J, Ganzhorn JU (1999) Feeding ecology of the hibernating primate *Cheirogaleus medius*: how does it get so fat? Oecologia 121:157–164. https://doi.org/10.1007/s004420050917
20. Britt A (2000) Diet and feeding behaviour of the black-and-white ruffed lemur (*Varecia* variegata variegata) in the Betampona Reserve, eastern Madagascar. Folia Primatol 71:133–141. https://doi.org/10.1159/000021741
21. Tan CL (1999) Group composition, home range size, and diet of three sympatric bamboo lemur species (genus *Hapalemur*) in Ranomafana National Park, Madagascar. Int J Primatol 20:547–566. https://doi.org/10.1023/A:1020390723639
22. Simmen B, Sauther ML, Soma T, Rasamimanana H, Sussman RW, Jolly A et al (2006) Plant species fed on by *Lemur catta* in gallery forests of the southern domain of Madagascar. In: Jolly A, Sussman JW, Koyama N, Rasamimanana H (eds) Ringtailed Lemur Biology: *Lemur Catta* in Madagascar. Springer, New York, pp 55–68
23. Sato H, Santini L, Patel ER, Campera M, Yamashita N, Colquhoun IC et al (2016) Dietary flexibility and feeding strategies of *Eulemur*: a comparison with *Propithecus*. Int J Primatol 37:109–129. https://doi.org/10.1007/s10764-015-9877-6
24. Campbell JL (2003) Malagasy primates: nutrition and gastrointestinal physiology with an emphasis on fiber utilization: Dissertation, North Carolina State University
25. McGrosky A, Meloro C, Navarrete A, Heldstab SA, Kitchener AC, Isler K et al (2019) Gross intestinal morphometry and allometry in primates. Am J Primatol 81:e23035. https://doi.org/10.1002/ajp.23035
26. Schwitzer C (2009) Gastrointestinal morphology of the crowned lemur (*Eulemur coronatus*). Anat Histol Embryol 38:429–431. https://doi.org/10.1111/j.1439-0264.2009.00964.x
27. Greene LK, McKenney EA (2018) The inside tract: the appendicular, cecal, and colonic microbiome of captive aye-ayes. Am J Phys Anthropol 66:960–967. https://doi.org/10.1002/ajpa.23481
28. Greene LK, Bornbusch SL, McKenney EA, Harris RL, Gorvetzian SR, Yoder AD et al (2019) The importance of scale in comparative microbiome research: new insights from the gut and glands of captive and wild lemurs. Am J Primatol 81:e22974. https://doi.org/10.1002/ajp.22974
29. Bolyen E, Rideout JR, Dillon MR, Bokulich NA, Abnet C, Al-Ghalith GA et al (2019) Reproducible, interactive, scalable, and extensible microbiome data science using QIIME 2. Nat Biotechnol 37:852–857. https://doi.org/10.1038/s41587-019-0209-9
30. Lozupone C, Lladser ME, Knights D, Stombaugh J, Knight R (2011) UniFrac: an effective distance metric for microbial community comparison. ISME J 5:169. https://doi.org/10.1038/ismej.2010.133
31. Douglas GM, Maffei VJ, Zaneveld JR, Yurgel SN, Brown JR, Taylor CM et al (2020) PICRUSt2 for prediction of metagenome functions. Nat Biotechnol 38:685–688. https://doi.org/10.1038/s41587-020-0548-6
32. RStudio Team (2021) RStudio: integrated development for R. RStudio, Inc., Boston, MA. http://www.rstudio.com. Accessed 8 Dec 2021
33. R Core Team (2021) R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. https://www.R-project.org/. Accessed 8 Dec 2021
34. Oksanen J, Blanchet FG, Friendly M, Kindt R, Legendre P, McGlinn D, et al (2020). vegan: community ecology package. R package version 2.5–7. https://CRAN.R-project.org/package=vegan. Accessed 8 Dec 2021
35. Arbizu PM (2017) PairwiseAdonis: Pairwise Multilevel Comparison using Adonis. R package version 0.1.1
36. Paradis E, Schliep K (2019) ape 5.0: an environment for modern phylogenetics and evolutionary analyses in R. Bioinformat 5:526–528. https://doi.org/10.1093/bioinformatics/bty633
37. Segata N, Izard J, Waldron L, Gevers D, Miropolsky L, Garrett WS et al (2011) Metagenomic biomarker discovery and

explanation. Genome Biol 12:R60. https://doi.org/10.1186/gb-2011-12-6-r60
38. Benjamini Y, Hochberg Y (1995) Controlling the false discovery rate: a practical and powerful approach to multiple testing. J Roy Stat Soc Series B (Methodological) 57:289–300
39. Biddle A, Stewart L, Blanchard J, Leschine S (2013) Untangling the genetic basis of fibrolytic specialization by Lachnospiraceae and Ruminococcaceae in diverse gut communities. Divers 5:627–640. https://doi.org/10.3390/d5030627
40. Goodwin S, Zeikus JG (1987) Physiological adaptations of anaerobic bacteria to low pH: metabolic control of proton motive force in *Sarcina ventriculi*. J Bacteriol 169:2150–2157. https://doi.org/10.1128/jb.169.5.2150-2157.1987
41. Shin N-R, Whon TW, Bae J-W (2015) Proteobacteria: microbial signature of dysbiosis in gut microbiota. Trends Biotechnol 33:496–503. https://doi.org/10.1016/j.tibtech.2015.06.011
42. Sachs G, Weeks DL, Melchers K, Scott DR (2003) The gastric biology of *Helicobacter pylori*. Annu Rev Physiol 65:349–369. https://doi.org/10.1146/annurev.physiol.65.092101.142156
43. Crespo-Piazuelo D, Estellé J, Revilla M, Criado-Mesas L, Ramayo-Caldas Y, Óvilo C et al (2018) Characterization of bacterial microbiota compositions along the intestinal tract in pigs and their interactions and functions. Sci Rep 8:12727. https://doi.org/10.1038/s41598-018-30932-6
44. Pérez-Pantoja D, Donoso R, Agulló L, Córdova M, Seeger M, Pieper DH et al (2012) Genomic analysis of the potential for aromatic compounds biodegradation in Burkholderiales. Environ Microbiol 14:1091–1117. https://doi.org/10.1111/j.1462-2920.2011.02613.x
45. Pimentel M, Gunsalus RP, Rao SS, Zhang H (2012) Methanogens in human health and disease. Am J Gastroenterol Supplements 1:28–33. https://doi.org/10.1038/ajgsup.2012.6
46. La Reau AJ, Suen G (2018) The Ruminococci: key symbionts of the gut ecosystem. J Microbiol 56:199–208. https://doi.org/10.1007/s12275-018-8024-4
47. Greene LK, Rambeloson E, Rasoanavio HA, Foss ED, Yoder AD, Drea CM et al (2021) Gut microbial diversity and ecological specialization in four sympatric lemur species under lean conditions. Int J Primatol. e-pub ahead of print. https://doi.org/10.1007/s10764-021-00257-9
48. Li Z, Si H, Nan W, Wang X, Zhang T, Li G (2019) Bacterial community and metabolome shifts in the cecum and colon of captive sika deer (*Cervus nippon*) from birth to post weaning. FEMS Microbiol Lett 366:fnz010. https://doi.org/10.1093/femsle/fnz010
49. Mancabelli L, Milani C, Lugli GA, Turroni F, Cocconi D, van Sinderen D, et al (2017) Identification of universal gut microbial biomarkers of common human intestinal diseases by meta-analysis. FEMS Microbiol Ecol 93:fix153. https://doi.org/10.1093/femsec/fix153
50. Zhang L, Wu W, Lee Y-K, Xie J, Zhang H (2018) Spatial heterogeneity and co-occurrence of mucosal and luminal microbiome across swine intestinal tract. Front Microbiol 9:48. https://doi.org/10.3389/fmicb.2018.00048
51. Dewar RE, Richard AF (2007) Evolution in the hypervariable environment of Madagascar. PNAS 104:13723–13727. https://doi.org/10.1073/pnas.0704346104
52. Blanco MB, Dausmann KH, Faherty SL, Yoder AD (2018) Tropical heterothermy is "cool": the expression of daily torpor and hibernation in primates. Evol Anthropol 27:147–161. https://doi.org/10.1002/evan.21588
53. McKenney EA, Maslanka M, Rodrigo A, Yoder AD (2018) Bamboo specialists from two mammalian orders (Primates, Carnivora) share a high number of low-abundance gut microbes. Microb Ecol 76:272–284. https://doi.org/10.1007/s00248-017-1114-8