Gut microbiota of ring-tailed lemurs (*Lemur catta*) vary across natural and captive populations and correlate with environmental microbiota

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Abstract

Background: Inter-population variation in host-associated microbiota reflects differences in the hosts' environments, but this characterization is typically based on studies comparing few populations. The diversity of natural habitats and captivity conditions occupied by any given host species has not been captured in these comparisons. Moreover, intraspecific variation in gut microbiota, generally attributed to diet, may also stem from differential acquisition of environmental microbes – an understudied mechanism by which host microbiomes are directly shaped by environmental microbes. To more comprehensively characterize gut microbiota in an ecologically flexible host, the ring-tailed lemur (*Lemur catta*; n = 209), while also investigating the role of environmental acquisition, we used 16S rRNA sequencing of lemur gut and soil microbiota sampled from up to 13 settings, eight in the wilderness of Madagascar and five in captivity in Madagascar or the U.S. Based on matched fecal and soil samples, we used source-sink ecological theory to examine covariation between the two types of consortia.

Results: The diversity of lemur gut microbes varied markedly within and between settings. Microbial diversity was not consistently greater in wild than in captive lemurs, indicating that this metric is not necessarily an indicator of host habitat or condition. Variation in microbial composition was inconsistent with either a single, representative gut community for wild conspecifics or a universal 'signal of captivity' that homogenizes the gut consortia of captive animals. Despite the similar, commercial diets of captive lemurs on both continents, lemurs within Madagascar were compositionally most similar, suggesting that non-dietary factors

govern some of the variability. In particular, soil microbial communities were most distinct between the two continents, and there was significant and context-specific covariation between lemur gut and soil microbiota.

Conclusions: As one of the broadest, single-species investigations of primate microbiota, our study highlights that gut consortia are sensitive to multiple scales of environmental differences. This finding begs a reevaluation of the simple 'captive vs. wild' dichotomy. Beyond the important implications for animal care, health, and conservation, our finding that environmental acquisition may mediate aspects of host-associated consortia further expands the framework for how host-associated and environmental microbes interact across different microbial landscapes.

Introduction

The structure of gut microbial communities within vertebrates is influenced in part by endogenous host factors, such as genotype and physiology[1–3], and in part by exogenous factors, such as sociality, seasonality, habitat quality, and diet[4–6]. These exogenous factors can influence which microbial taxa in a gut community thrive or become depauperate, as amply demonstrated in dietary studies[7–10], or they can provide opportunities for more direct routes of microbial acquisition[11–14]. For example, the transmission of microbes between hosts, including horizontal pathogen transfer[15–17] or vertical transmission during the birthing process and nursing[18,19], are significant drivers of host health and development. There is, likewise, the potential for horizontal acquisition of microbes via exposure to environmental consortia on natural (e.g., soil) and man-made surfaces, plus on food and in water[12,20–23]; however, this latter route to shaping host-associated communities, hereafter referred to as 'environmental acquisition,' remains understudied. Here, we match-sampled ring-tailed lemur (*Lemur catta*) feces and soil from 13 settings, representing both a large portion of the lemurs' natural habitat range in Madagascar and a range of captive housing conditions in Madagascar and the U.S. (Table 1), to (a) characterize variation in host gut microbiota, (b) characterize variation in soil microbiota, and (c) test for any covariation between host and soil communities. Examining environmental microbes alongside host-associated communities is a first step to understanding the role of environmental acquisition in population-level differences between host microbiomes.

Previous studies of intraspecific variation in gut microbiota, often framed using a 'wild vs. captive' comparison, have provided valuable descriptions of differences in presumed extremes[24–26]. For example, researchers often report a 'signal of captivity,' whereby the gut microbiota of captive hosts differ significantly from those of wild conspecifics, converging on a perturbed or

80 ‘humanized’ composition[25,27,28]. Perturbations of this nature are generally attributed to
81 commercial diets that include manufactured chow and cultivated produce[27,29,30]; nevertheless,
82 studies of captive populations have been focused on accredited zoos or rescue facilities that may
83 not represent the range of captive conditions or may be confounded by within-species
84 comparisons across continents[26,29,31]. Even comparative field studies have been limited in the
85 number of populations per species studied, typically to a few populations that differ on a given
86 metric of interest (e.g. season, habitat type or quality[32–34]). Because hosts experience a wider
87 range of environmental settings than is typically encompassed within wild vs. captive
88 comparisons, a broader comparative approach is necessary to provide a more comprehensive and
89 nuanced understanding of gut microbiota variation.

90 As noted, differential exposure to environmental microbes provides potential for horizontal
91 transmission and environmental acquisition[20,22,23,35–37], with the ingestion of specific microbes
92 being linked to novel digestive functions of the gut microbiota[38–40]. Under certain conditions,
93 environmental acquisition has been shown to outweigh vertical transmission as the main mode of
94 microbial colonization[41,42]. Although environmental acquisition may promote heterogeneity
95 within and between hosts[43], its role rarely has been considered a differentiating factor between
96 wild and captive hosts. Husbandry practices and veterinary care, for example, introduce cleaning
97 products and antibiotics to the microbial environment of captive animals[44,45], further
98 differentiating it from natural habitats[46], with potentially critical consequences to microbiome
99 structure and function.

100 Ring-tailed lemurs are semi-terrestrial, omnivorous strepsirrhine primates[47,48] that occupy
101 various habitats across southern Madagascar[49] and also survive well in captivity[50]. Their
102 ecological flexibility, coupled with existing knowledge about their gut microbiome[26,51–53],

motivates broader comparative study of intraspecific variation that takes environmental acquisition of microbes into consideration. Here, we combine 16S rRNA sequencing and statistical tools based on source-sink ecological theory[54] to analyze covariation between lemur gut microbiota (e.g., sink communities) and soil microbiota (e.g., source communities). Given the variability of environmental factors across our multiple settings, we expect the diversity, membership, and composition of lemur gut microbiota and soil microbiota to differ within and between three, broad 'environmental' conditions: wilderness, captivity in Madagascar, and captivity in the U.S (Table 1).

If diet or habitat quality were the main driver of gut microbiota composition, we would expect (a) wild lemurs to show variation between their natural settings, (b) captive lemurs, regardless of continent, to show similar gut microbiota between their settings (reflecting commercial diets and perturbed habitats), and (c) wild and captive lemurs to differ most drastically from one another, in line with prior studies[27]. If, however, environmental acquisition were to play a major role in shaping lemur gut microbiota, we would again expect (a) wild lemurs to show variation between their natural settings (reflecting the soil microbiota of the lemurs' habitat), but we would expect (b) Malagasy lemurs (wild and captive) to share certain soil-derived microbiota, differing most drastically from lemurs in captivity in the U.S., and (c) differential access to soil within captive conditions to correlate with differential soil-associated microbes present in hosts. With regard to the latter, for example, we might expect greater proportions of soil-associated microbes in captive lemurs that gain access to natural enclosures compared to their counterparts that are housed indoors.

Results

126

127 *Lemur gut microbiota: Variation in diversity, membership, and composition*

128 Alpha diversity. Across the gut microbiota of all ring-tailed lemurs sampled in this study,
129 metrics of alpha diversity differed significantly by environmental condition (Generalized Linear
130 Models or GLM; Shannon: $F = 23.773$, $p < 0.001$; Faith's phylogenetic: $F = 4.415$, $p = 0.013$;
131 Figures 1a, b) and by setting (GLM; Shannon: $F = 13.157$, $p < 0.001$; Faith's phylogenetic: $F =$
132 5.628, $p < 0.001$; Figures 1c, d; Supplementary Material 1). The gut microbiota of wild lemurs
133 and captive lemurs in the U.S. (hereafter, 'captive$_{U.S.}$ lemurs') were similarly diverse overall
134 (pairwise Wilcoxon test; Shannon: $p = 0.635$; Faith's phylogenetic: $p = 0.056$; Figures 1a, b),
135 whereas those of captive lemurs in Madagascar (hereafter, 'captive$_{M}$ lemurs') were significantly
136 less diverse (pairwise Wilcoxon test; Shannon, wild vs. captive$_{M}$ lemurs: $p < 0.001$; wild vs.
137 captive$_{U.S.}$ lemurs: $p < 0.001$; Faith's phylogenetic, wild vs. captive$_{M}$ lemurs: $p = 0.022$; wild vs.
138 captive$_{U.S.}$ lemurs: $p = 0.021$; Figures 1a, b). Within environmental condition, however, both
139 metrics of alpha diversity varied widely between the different settings (Figures 1c, d;
140 Supplementary Material 1). For example, among wild lemurs, setting was a significant predictor
141 of both metrics of alpha diversity (GLM; Shannon diversity: $F = 20.768$, $p < 0.001$; Faith's
142 phylogenetic: $F = 11.104$, $p < 0.001$). Sex was not a significant predictor in any models of either
143 alpha diversity metric (Supplementary Material 1).

144 Community membership. The membership of lemur gut microbiota included 64 abundant
145 taxa (i.e., those that accounted for >1% of sequences). Of these 64 taxa, only four (6.2%) were
146 shared across lemurs from all settings: the genera *Bacteroides* (phylum Bacteroidetes),
147 *Rikenellaceae RC9 gut group* (Bacteroidetes), *Erysipelotrichaceae* UCG-004 (Firmicutes), and
148 *Treponema 2* (Spirochaetes). Within condition, five (7.8%) taxa were shared by all wild lemurs,

149 whereas 10 (15.6%) and six (9.4%) taxa were shared by captive$_{M}$ and captive$_{U.S.}$ lemurs,
150 respectively (Figure 2). Using Analysis of Compositions of Microbiomes (ANCOM), we
151 identified 801 amplicon sequence variants (ASVs) that were differentially abundant across the
152 three conditions. For example, members of the Erysipelotrichaceae family characterized the
153 microbiota of wild lemurs, whereas taxa from the Spirochaetaceae and Prevotellaceae families
154 were more abundant in the gut microbiota of captive lemurs from both continents.
155 *Erysipelotrichaceae UCG-004* and *Treponema 2*, for example, were abundant in all lemurs
156 (Figure 2), but the log ratios of the two genera distinguished lemur gut microbiota by the three
157 environmental conditions and, in particular, differentiated wild lemurs from captive$_{U.S.}$ lemurs
158 (Figure 3).
159 Beta diversity. The composition of lemur gut microbial communities was significantly
160 distinct across the three environmental conditions, as revealed by beta diversity (Permutational
161 Multivariate Analysis of Variance or PERMANOVA; wild vs. captive$_{M}$ lemurs: pseudo-F =
162 30.169, $p < 0.001$; wild vs. captive$_{U.S.}$ lemurs: pseudo-F = 97.912, $p < 0.001$; captive$_{M}$ vs.
163 captive$_{U.S.}$ lemurs: pseudo-F = 20.808, $p < 0.001$). Across all subjects, gut microbiota
164 composition clustered distinctly by condition (principal coordinate analysis of unweighted
165 UniFrac distances; Figures 4a, b). One notable exception, however, owed to a single pet lemur:
166 Unlike its in-country peers (i.e., other captive$_{M}$ lemurs), its microbial community structure
167 matched those of wild lemurs (see arrows in Figures 4a, b).
168 Across the three environmental conditions, Random Forest Analysis accurately assigned 208
169 of the 209 gut microbial profiles to the correct condition, with a low (0.48%) out-of-bag (OOB)
170 error rate. Based on its gut microbiota, only the previously mentioned pet lemur (see arrows in
171 Figure 2a, b) was misclassified as a wild lemur. Across the 13 settings, Random Forest Analysis

accurately classified 189 of the 209 microbial profiles (OOB error = 9.57%). The gut microbial communities of wild and captive lemurs in Madagascar were misclassified at rates of 7.9% and 7.3%, respectively, whereas those of captive lemurs in the U.S. were misclassified at a rate of 20.6%.

With respect to uniformity within environmental condition, the composition of gut microbial communities were least dissimilar between wild lemurs and most dissimilar between $captive_M$ lemurs (Kruskal-Wallis test; main effect of condition on beta diversity: $\chi^2 = 27487$, $p < 0.0001$; pairwise Wilcoxon test; within wild vs. within $captive_M$ lemurs: $p < 0.001$; within wild vs. within $captive_{U.S.}$ lemurs: $p < 0.0001$; Figure 4c). Between conditions, the microbiota of wild and $captive_M$ lemurs were the least dissimilar, whereas the microbiota of wild vs. $captive_{U.S.}$ lemurs were the most dissimilar (pairwise Wilcoxon test: 'wild vs. $captive_M$' vs. 'wild vs. $captive_{U.S.}$', $p < 0.0001$; 'wild vs. $captive_M$' vs. '$captive_M$ vs. $captive_{U.S.}$', $p < 0.0001$; Figure 4c). Considering wild lemurs only, microbiota composition clustered by setting (Figure 4d). Although there was some overlap between settings, the patterns are suggestive of microbial 'signatures' across different settings.

Soil microbiota: Variation in diversity, membership, and composition

<u>Alpha diversity</u>. Across the eight settings for which we sampled soil, the alpha diversity of soil microbiota did not vary significantly between conditions (Kruskal-Wallis test; Shannon diversity: $\chi^2 = 3.3457$, $p = 0.187$; Faith's phylogenetic: $\chi^2 = 3.433$, $p = 0.179$; Figure 5) nor between settings (Kruskal-Wallis test; Shannon diversity: $\chi^2 = 7.496$, $p = 0.379$; Faith's phylogenetic: $\chi^2 = 8.936$, $p = 0.257$; Figure 5). These null findings may owe to small sample sizes.

195 Community membership. The membership of soil communities included 77 abundant taxa,
196 of which none were shared across all settings (Figure 6). Of the identified soil microbiota,
197 78.12% were unique to the soil samples and were not found in any lemur fecal samples. For the
198 five wild populations for which we sampled soil, only five abundant taxa were shared: the genera
199 *Bacillus* (phylum Firmicutes), *Steroidobacter* (Proteobacteria), *Bryobacter* (Acidobacteria), and
200 *RB41* (Acidobacteria), and an unidentified member of the class Subgroup 6 (Acidobacteria).
201 ANCOM identified nine ASVs that were differentially abundant across all soil samples, five of
202 which (55.6%) belonged to the Balneolaceae family. In addition, compared to soil from
203 Madagascar, the soil communities from the U.S. (hereafter, captivity$_{U.S.}$ soil) were differentially
204 enriched for the genus *Bacillus*. By contrast, members of the family Nitrososphaeraceae
205 (Thaumarchaeota) and the genus *Acinetobacter* (Proteobacteria) characterized soil from the
206 wilderness and captivity settings in Madagascar (hereafter, wilderness and captivity$_{M}$ soils,
207 respectively; Figure S1).
208 Beta diversity. The beta diversity of the soil microbiota varied between conditions (Figure 7),
209 but only significantly so between wilderness and captivity$_{M}$ soils (PERMANOVA; wilderness
210 vs. captivity$_{M}$ soils: pseudo-F = 1.337, p = 0.202; wilderness vs. captivity$_{U.S}$ soils: pseudo-F =
211 3.897, p = 0.012; captivity$_{M}$ vs. captivity$_{U.S}$ soils: pseudo-F = 7.752, p = 0.329). Variation in soil
212 communities within a condition was not significantly different between wilderness soils and
213 captivity$_{U.S.}$ soils (pairwise Wilcoxon test, p = 0.130; Figure 7c). Between conditions, wilderness
214 and captivity$_{M}$ soils had the lowest dissimilarities (pairwise Wilcoxon test; 'wilderness vs.
215 captivity$_{M}$' vs. 'wilderness vs. captivity$_{U.S}$' soils: $p < 0.001$; 'wilderness vs. captivity$_{M}$' vs.
216 'captivity$_{M}$ vs. captivity$_{U.S}$': p = 0.016; 'wild vs. captivity$_{U.S}$' vs. 'captivity$_{M}$ vs. captivity$_{U.S}$': p =
217 0.338 Figure 7c).

218

219 *Covariation of lemur gut and soil microbiota*

220 There were 191 ASVs shared between lemur gut and soil microbiota. These were dominated
221 by members of the Firmicutes (75 ASVs or 39.3%), Proteobacteria (49 ASVs or 25.6%), and
222 Bacteroidetes (38 ASVs or 19.9%) phyla. Although many of the shared taxa were abundant
223 (>1%) in either lemur gut or soil microbiota, only one genus, *Acinetobacter* (Proteobacteria), was
224 abundant in both lemur gut and soil microbiota.

225 As would be predicted if environmental acquisition impacts host microbial communities,
226 there was a significant correlation between the abundances of microbes in lemur feces and soil
227 samples (Mantel test; $r = 0.494$, $p < 0.001$). The proportion of 'soil-associated' microbes found
228 in lemur gut microbiota varied significantly across conditions (Kruskal-Wallis test; $\chi^2 = 73.862$,
229 $p < 0.001$; Figure 8a) and settings (Kruskal-Wallis test; $\chi^2 = 112.69$, $p < 0.001$; Figure 8b).
230 Overall, the gut microbiota of wild lemurs had significantly greater proportions of soil-associated
231 microbes compared to those of all captive lemurs (pairwise Wilcoxon test, $p < 0.001$; Figure 8).
232 In addition, $\text{captive}_{\text{M}}$ lemurs had significantly greater proportions of soil-associated microbes in
233 their gut microbiota compared to $\text{captive}_{\text{U.S.}}$ lemurs (pairwise Wilcoxon test; $p < 0.001$; Figure
234 8). For lemurs housed at the DLC, those that semi-free-ranged in outdoor, natural habitat
235 enclosures had significantly greater proportions of soil-associated microbes in their gut
236 microbiota compared to lemurs that did not have access to forested enclosures (Kruskal-Wallis
237 test; $\chi^2 = 4.641$, $p = 0.031$; Figure 8c).

238 Soil from within a lemur's setting accounted for, on average, significantly greater proportions
239 of the lemur's gut microbiota than did soil communities from other settings (Figure 9,
240 Supplementary Material 2). Overall, the greatest proportion of soil-associated microbes within

241 lemur gut microbiota occurred when the lemurs and soil were both from the wilderness (Figure
242 9; Supplementary Material 2). The proportion of soil-associated microbes in lemur gut
243 microbiota dropped to near zero when comparing the lemur gut and soil microbiota between
244 samples from the wilderness and those from captivity in the U.S. (Figure 9; Supplementary
245 Material 2).
246
247 **Discussion**
248 Through extensive fecal and soil sampling from multiple settings representing the ring-
249 tailed lemurs' natural range in Madagascar and in captivity on two continents, we have
250 highlighted (1) the wide and often underrepresented variety of gut microbiota present within a
251 single host species, (2) the lack of a universal 'signal of captivity' that uniformly decreases
252 microbial diversity, (3) aspects of microbiota membership and composition that differ markedly
253 between wild and captive populations, and (4) covariance between lemur gut and soil microbiota,
254 which points to a key role of environmental microbes. Researchers have reported host 'group
255 signatures' in microbiota, often attributed to the social transmission of microbes[5,55–58]; our results
256 expand this concept to 'population signatures' and draw attention to the potential role of
257 environmental acquisition of microbes in mediating significant inter-population variation.
258 Across populations of wild lemurs, we first observed substantial variation in gut microbiota
259 diversity, membership, and composition, indicating that there is not a single 'representative' gut
260 community for wild ring-tailed lemurs, as is likely the case for most host species. Nonetheless,
261 the pattern of natural variation observed did not always meet expectations. For example, lemurs
262 living in what is considered a relatively 'pristine' site, IVO – a recently discovered humid forest
263 patch that is relatively undisturbed by human activity – unexpectedly had the second-lowest

264 diversity of gut microbes. To the extent that lack of disturbance is a proxy of habitat quality, this
265 pattern would be inconsistent with previous reports that greater habitat quality promotes more
266 diverse gut microbiota[59,60]. In prior studies, the gut microbiota of ring-tailed lemurs were
267 relatively unaffected by habitat degradation[52]. Therefore, either pristine habitats can be of low
268 quality or the ecological and dietary flexibility of this species may dampen the impact of
269 variation in habitat quality and type, relative to more specialized primates (e.g., folivores)[26,61–63].
270 That we found significant, natural, inter-population variation in a relatively hardy and robust
271 species[49,64] suggests that hosts with greater sensitivity to environmental variation, including
272 habitat quality and type, would likely show even greater variation than that described herein. If
273 so, studies constrained to single or few host populations are likely to underrepresent the wide-
274 scale, natural variation in host gut microbiota.

275 We next observed gut microbiota to be compositionally distinct across populations of captive
276 lemurs. Contrary to many previous studies[65–68], but consistent with others[69–72], the gut microbiota
277 of captive lemurs were not consistently less diverse than those of wild lemurs nor were they
278 compositionally homogenized by the similar commercial diets provided to captive subjects[67,73].
279 Heterogeneous gut microbiota could reflect slight differences in the diets provided (as the
280 produce and browse available differ between captivity settings), but such minor dietary variation
281 is unlikely to be the sole driver of such marked microbial differences, particularly in an
282 omnivorous host. Non-dietary factors must have contributed to distinguishing the gut
283 communities of captive lemurs. Indeed, the gut microbiota of captive lemurs in Madagascar were
284 compositionally more similar to those of their wild counterparts than to those of captive lemurs
285 in the U.S. Based on this observation, we suggest that the effect of a commercial diet is not
286 necessarily the strongest differentiator of gut consortia and that the effects of captivity cannot be

287 standardized across populations. Our results raise questions about the commonly held view that
288 greater alpha diversity is both (a) a hallmark of wild individuals and (b) a proxy for a healthier
289 gut community[74–78]. Although we did not assess gut health, pet lemurs fed diets of rice and fruit,
290 living in close contact with people and domestic animals, often housed solitarily indoors, are
291 prone to disease[79–81]; yet, their gut consortia were as diverse as those of wild lemurs living at the
292 relatively pristine site, IVO. Moreover, captive lemurs from the DLC and NCZ in the U.S. had
293 some of the most diverse gut consortia, equaling the greatest diversity seen in wild lemurs (e.g.,
294 in BEZ lemurs). These results add to the mounting evidence[61,82,83] that alpha diversity alone,
295 without the context of host ecology and other microbiome data, should not be used to extrapolate
296 about the health state of gut consortia or the host's environment.

297 We also found that, between wild and captive lemurs, the membership and composition of
298 gut microbiota was indicative of the environmental condition. There was little evidence of a
299 diverse 'core' microbiome, as only four taxa were found to be abundant across all lemur
300 populations. Two of those core taxa, *Erysipelotrichaceae UCG-004* and *Treponema 2*, were
301 differentially abundant between the three conditions. Despite links between members of
302 *Erysipelotrichaceae* and high-fat, commercial diets in humans[84], *Erysipelotrichaceae* microbes
303 were reported to be enriched in wild compared to captive chimpanzees[85], mirroring our findings
304 in lemurs. Furthermore, the genus *Erysipelotrichaceae UCG-004* was more abundant in the gut
305 microbiota of chimpanzees, relative to humans[86], and in folivorous woolly lemurs compared to
306 other lemur species[87]. The functionally diverse members of the *Treponema* genus were more
307 abundant in the gut microbiota of captive vs. wild hosts in other species[85,88]. *Treponema*
308 members break down pectin[89,90], a complex plant polysaccharide enriched in ripe fruits, such as
309 those commonly provided to captive ring-tailed lemurs[91,92]. Compositionally, the gut microbiota

310 of wild lemurs were markedly less varied than those of lemurs in all captivity settings. These
311 findings support the "Anna Karenina" principle[93,94], which posits that perturbations of microbiota
312 result in unstable communities and, thus, 'unperturbed' hosts have less variation in their
313 microbiota than do 'disturbed' hosts. A single exception to the gut microbiota clustering
314 according to the hosts' conditions was a pet lemur with gut microbiota that resembled that of
315 wild lemurs. Although we can only speculate about this individual's history, if recently taken
316 from their natural habitat, the gut microbiota could still reflect the wild origins of this animal,
317 potentially indicative of gradual change in response to environmental shifts[95,96].
318 Lastly, we observed that patterns in lemur gut microbiota were somewhat mirrored in the
319 diversity and composition of soil microbiota, suggesting that environmental conditions other than
320 diet, including exposure to external microbes in soils, may influence gut microbiomes[97].
321 Madagascar's geographical isolation for ~88 million years accounts for high levels of floral and
322 faunal endemism[98,99]. The same is true of microbes, as evidenced by the numerous, unique
323 pathogenic microorganisms found on the island[100–103]. Unsurprisingly, therefore, soil microbiota
324 in Madagascar, whether originating in wilderness or captivity settings, were similar in
325 composition and significantly divergent from soils in the U.S.[104]. Given the disparate geographic
326 distributions of many wild vs. captive animals, environmental acquisition that reflects local
327 microbial endemism may be particularly relevant for distinguishing gut microbiota between wild
328 and captive conspecifics. For example, the natural ranges of most primates occur in the
329 tropics[105,106], yet most accredited zoos and captive facilities that house primates are found outside
330 of tropical regions (in e.g., Europe and North America)[107,108]; the distinct environmental
331 consortia surrounding wild and captive conspecifics should reflect their geographic or
332 continental divides.

333 Regarding the exposure to environmental microbes, soil-associated microbes were more
334 prevalent in lemurs that had greater exposure to natural environments and the acquired soil
335 microbes were specific to the lemurs' environment, reflecting active environmental acquisition.
336 This observation expands on findings that abiotic soil properties mediate primate gut
337 microbiota[97]. Wild and captive ring-tailed lemurs perform geophagy (i.e., earth-eating), a
338 behavior that is linked to nutrient and microbial supplementation[109,110] and is a potential vector
339 for the incorporation of environmental microbes[39]. Similarly, dietary items may act as vessels of
340 soil or environmental microbes[40]; dietary variation across wild and captive lemurs may influence
341 gut microbiomes by simultaneously offering different nutrients and different microbes. Akin to
342 most cross-sectional studies of microbiomes, we were unable to assess the persistence or
343 viability of the soil-associated microbes in lemur gut communities. It is, therefore, possible that
344 the soil-associated microbes in lemur guts were ephemeral or non-viable; however, our results
345 indicate setting-specific, environmental acquisition, supporting that these patterns are not random
346 and that the acquired microbes may be subject to filters that enable the incorporation of only
347 specific microbes[20,111,112]. Furthermore, we analyzed these data from the perspective that
348 environmental consortia act as sources of microbes for host-associated communities, but we
349 expect consistent, bidirectional transmission of microbes between hosts and their environments,
350 a relationship that warrants further investigation.
351 While expanding our understanding of the factors that shape host-microbe relationships,
352 these results also have significant potential to inform animal care and conservation strategies.
353 Perturbed microbiota are increasingly recognized as culprits of obesity, gastrointestinal distress,
354 and even associated mortality in captive animals[113–115,75]. Given that lemurs are among the most
355 endangered mammals on the planet[116], maintaining populations of healthy animals in captivity is

356 an important 'safety net' that augments *in-vivo* conservation efforts[117,118]. We suggest that
357 environmental acquisition may be a key component of 'rewilding' or 'bioaugmenting' captive
358 animal gut microbiota, a process by which gut consortia can be reshaped to better promote host-
359 microbe symbiosis[26,117,119]. Identifying what comprises healthy gut microbiota is a complex and
360 ongoing area of research; nonetheless, we show that environmental acquisition is a potential
361 driver of microbial communities and thus should be considered as a component of animal health.
362

363 **Conclusions**

364 Even in a relatively robust, omnivorous host, gut microbiota are distinct across populations.
365 This variation reflects environmental variability that is underrepresented by a simple wild vs.
366 captive dichotomy. Moreover, concurrent analysis of lemur gut and soil microbiota supports the
367 premise that environmental acquisition contributes to shaping host-associated microbiota; hosts
368 and their associated microbes are components of a larger landscape that includes interactions
369 with environmental microbes. Together, these results expand our understanding of intraspecific
370 host-microbe dynamics under varying environmental conditions and reinforce the value of
371 broad-scale, comparative investigations of microbial variation within a single host species.
372

373 **Methods**

374 *Study sites*

375 Our research sites included 13 settings (one per 'population'), grouped under the following
376 three environmental conditions: wilderness in Madagascar (8 settings), captivity in Madagascar
377 (2 settings), and captivity in the U.S. (3 settings; Table 1). The wilderness settings occurred in
378 protected areas (e.g., national parks, community-managed reserves) that varied in habitat type

379 (Table 1). The captivity settings in Madagascar included the Lemur Rescue Center (LRC;
380 Toliara, Madagascar), where the animals were socially housed, and various townships that were
381 home to individual pets. Lastly, the captivity settings in the U.S. included the North Carolina
382 Zoo (NCZ; Asheboro, NC), the Duke Lemur Center (DLC; Durham, NC), and the National
383 Zoological Park (NZP; Washington, DC). These facilities were comparable to one another, all
384 with socially housed lemurs.
385
386 *Subjects*
387 Across all research sites, our subjects included 215 adult, ring-tailed lemurs (82 male, 81
388 female, 52 of unknown sex; Table 1). The wilderness sites were each occupied by multiple lemur
389 troops, ranging in size from 5-24 individuals. Excluding the pets, all captive settings included
390 groups of 2-7 lemurs that had access to indoor and outdoor enclosures, and were provided
391 facility-standardized diets (i.e., fresh produce and commercial chow, freely available water).
392 Certain animals at the LRC and the DLC also had access to natural habitat enclosures that,
393 respectively, consisted of dry and spiny forest (LRC) or North American deciduous and pine
394 hardwood forest (DLC). The pets were kept in human dwellings (i.e., houses or hotels) and were
395 fed fruit, rice, and other foods intended for human consumption.
396
397 *Sample collection*
398 During a span of four years (2016-2020), we collected 'matched' fecal and soil samples from
399 our subjects and study sites, respectively. Within 8 weeks of fecal or soil collection, the samples
400 were transported to the U.S., where they were stored at -80 °C, until analysis.

401 For feces, we opportunistically collected fresh samples, upon the lemur's observed voiding.
402 In Madagascar, collections occurred during the dry season (May-October) and, in the U.S.,
403 collections occurred end of summer through fall (August-November). To avoid soil
404 contamination of the fecal sample, we removed the outer layer of each fecal pellet. We then
405 placed the sample in an Omnigene tube that contained a stabilizing buffer that preserved
406 microbial communities at room temperature for 8 weeks (Omnigene.Gut tube, DNAgenotek,
407 Ontario, Canada[120,121]). All settings were represented by fecal samples from minimally two
408 lemurs (the maximum number of individuals represented was 33).
409 When collecting soil in nature, we avoided high-defecation areas (e.g., under sleeping trees)
410 while identifying core areas where lemurs most commonly spent time on the ground. Within
411 these core areas, we demarcated a 2-3 m^2 area and collected soil from each of five evenly spaced
412 locations, using a clean, individually wrapped, sterile plastic spatula. For each area, the five
413 aliquots of topsoil (top 2-3 cm of soil) were pooled in a single Omnigene tube to create a
414 representative soil sample. Because multiple lemur troops inhabited each of the wilderness
415 settings, in some cases with overlapping core areas, we prioritized collecting soil samples from
416 areas of maximal use. In some cases, we were unable to collect soil samples for every troop that
417 provided fecal samples. At the LRC and DLC, we used the same collection methods to collect
418 soil samples from areas in the natural habitat enclosures where lemurs semi-free-ranged. Because
419 it is illegal to own pet lemurs in Madagascar, we minimized owner concern by collecting only
420 fecal samples for this group. Because of other logistical and analytical constraints (see below),
421 only eight of the 13 settings were represented by usable, pooled soil samples.
422
423 *Microbial DNA extraction and sequencing*

424 Following the manufacturer's protocols for the DNeasy Powersoil kit (QIAGAN, Frederick,
425 MD), we extracted bacterial genomic DNA from fecal and soil samples. We quantified DNA
426 using a Fluorometer (broad-spectrum kit, Qubit 4, Thermo Fisher Scientific, Waltham, MA).
427 Aliquots of extracted DNA were sent to Argonne National Laboratory's Environmental
428 Sequencing facility (Lemont, IL) for library preparation and amplicon sequencing of the 16S
429 rRNA gene. After amplification of the V4 region with region-specific primers and sample-
430 specific 12-base barcodes, samples were pooled and amplicon libraries were cleaned using
431 AMPure XP Beads. Amplicons were then sequenced on a 151 x 151 base pair Illumina MiSeq
432 run[122].
433
434 *Bioinformatics and statistics*
435 We processed the raw sequence data using a previously published bioinformatics pipeline
436 generated in QIIME2[123]. In brief, we used the pipeline to join forward and reverse reads,
437 demultiplex and quality filter the joined reads (DADA2), generate a phylogenetic tree, and
438 assign taxonomy based on 99% sequence similarity (SILVA database [124,125], ver. 138.1). After
439 quality filtering, samples with fewer than 10,000 sequences were removed from downstream
440 analyses, resulting in 209 fecal samples and 25 soil samples with over 11 million combined reads
441 and an average of ~50,000 reads per sample. To visually represent rare taxa that had relative
442 abundances < 1% of the total sequences, we combined them into the conglomerate "Other"
443 category (Figures 1 and 6). Using tables of amplicon sequence variants (ASVs), we calculated
444 metrics of alpha diversity (Shannon and Faith's Phylogenetic diversity metric) and beta diversity
445 (UniFrac distances).

446 To test for differences in alpha diversity between the gut microbiota of lemurs under the
447 three environmental conditions and in the 13 settings, we first used generalized linear models
448 (GLMs; glm in R, ver, 4.0.2) with condition or setting and sex as fixed effects. To further test for
449 variation in lemur gut microbiota and soil microbiota alpha diversity, we used nonparametric
450 statistics (e.g., Kruskal-Wallis tests, and pairwise Wilcoxon rank sum tests with Benjamini-
451 Hochberg adjustment) to perform pairwise comparisons between the various conditions and
452 settings. To identify and test for effects of condition or setting on beta diversity (unweighted
453 UniFrac distances) in lemur fecal and soil microbiota, we used principal coordinate analysis (i.e.,
454 to visualize clustering of microbiota composition) and Permutational Multivariate Analysis of
455 Variance (PERMANOVA) in QIIME2. We then performed Random Forest Analysis[126], which is
456 a supervised learning technique that uses decision trees to classify data to specific categories and
457 provides an overall model error rate (out of the bag error or OOB error). To identify microbes
458 enriched in specific groups of samples, we used differential abundance analyses via Analysis of
459 Compositions of Microbiomes (ANCOM) and songbird software[127] in QIIME2, paired with
460 visualization through Qurro[128].
461 For the eight settings where we obtained matched fecal and soil samples (Table 1), we
462 analyzed covariation between lemur gut microbiota and the associated soil communities by
463 performing a Mantel test on microbial abundance matrices of lemur gut and soil microbiota.
464 Because multiple lemur fecal samples were associated with each soil sample, we created
465 comparable matrices for the Mantel test by averaging the microbial abundances across the fecal
466 samples of lemurs directly associated with a given soil sample, resulting in a single, mean lemur
467 gut community associated with each soil community. For this process, we omitted fecal samples
468 from troops not represented by a soil sample or for which troop identity was unknown.

469 To test if soil-associated microbes were present in lemur gut microbiota, we used FEAST, a
470 tool for fast expectation-maximization microbial source tracking[129]. FEAST assumes each 'sink'
471 sample is a convex combination of known and unknown 'sources' and uses multinomial
472 distributions and machine-learning classification to model the microbial source-sink data[129]. For
473 this analysis, we used the matched lemur gut and soil samples; all soil samples collected in a
474 given setting were used to represent the potential exposure to environmental microbes
475 experienced by all sampled lemurs in that same setting, regardless of troop identity. Because we
476 were testing whether environmental acquisition influences lemur gut microbiota, and because
477 this analysis requires an assumption of directionality (i.e., from a source to a sink), we
478 categorized soil samples as 'sources' and lemur fecal samples as 'sinks'; however, we
479 acknowledge and discuss the potential for bi-directional transmission of microbes between
480 lemurs and soil. For each lemur fecal sample, we calculated the proportions of microbes that
481 were identified as stemming from each soil community and from a default 'unknown source' that
482 accounts for microbes not relevant to soil microbiota. Lastly, we used FEAST to test for
483 differences in the proportion of soil microbes in the gut microbiota of lemurs at the DLC that
484 were either semi-free-ranging or sequestered to indoor enclosures.
485
486 **Declarations**
487 *Ethics approval and consent to participate*
488 Sampling in Madagascar occurred with approval from Madagascar National Parks and
489 appropriate governmental agencies (Ministry of Environment, Ecology, and Forests; permit #s
490 147/18/MEEF/SG/DGF/DSAP/SCB.Re, 152/19/MEDD/SG/DGEF/DGRNE,
491 159/16/MEEF/SG/DGF/DSAP/SCB.Re, 154/17/ MEEF/SG/DGF/DSAP/SCB.Re,

156/19/MEEF/SG/DGF/DSAP/SCB.Re). Sampling at the DLC, NCZ, and NZP occurred with approval from the appropriate Animal Care and Use Committees (Duke University's Institutional Animal Care and Use Committee: protocol #A111-16-05; North Carolina Zoo Animal Care and Use Committee: approved without protocol number; Smithsonian National Zoological Park Research Animal Care and Use Committee: approved without protocol number).

Consent for publication

Not applicable. This study does not contain any individual person's data in any form.

Availability of data and material

Sequencing reads are available in the National Center for Biotechnology Information's Sequence Read Archive (BioProject ID #TBD, BioSample accession #s TBD). Additional datasets generated and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Competing interests

We attest that no author has financial or non-financial competing interests.

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Authors' contributions

SLB and CMD conceived of the study, with input from LKG. SLB, LKG, SR, SC, RSR, TAC, and ML collected samples, documented metadata, and transported materials/samples. SLB

514 performed the bioinformatic and statistical analyses. SLB and CMD wrote the manuscript and all
515 authors read and approved the submitted version.

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525

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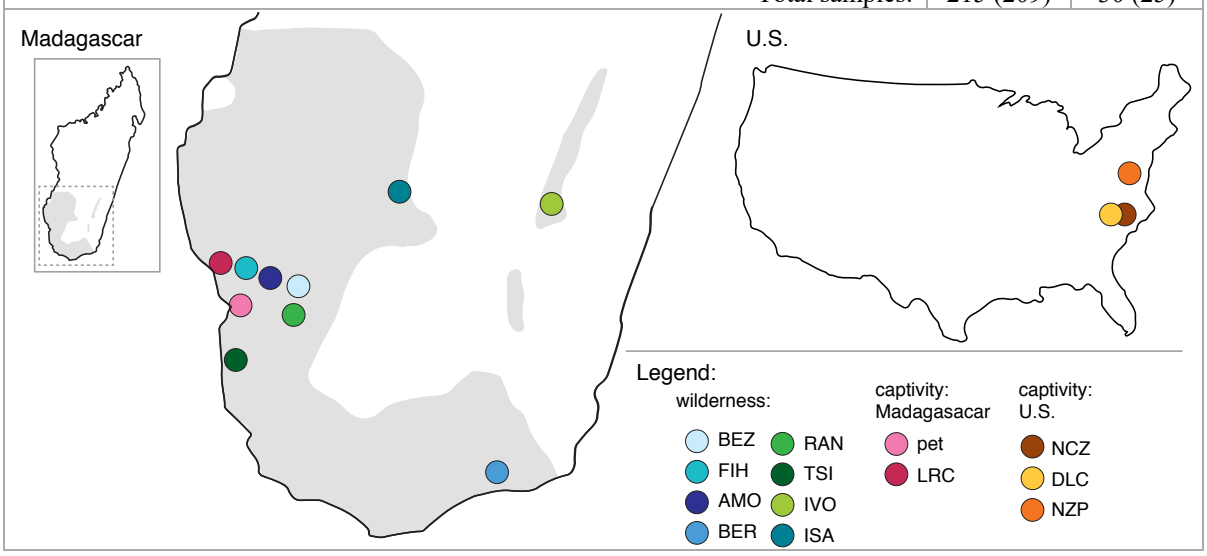
Table 1. Research settings (names, descriptions, and locations) and samples collected under wilderness conditions and under captivity conditions in Madagascar and the U.S. A subset of the samples collected were omitted from analyses owing to low-yield extractions or low-quality sequencing. Soil samples could not always be obtained. Maps show locations of each setting; the gray shaded area shows the natural range of wild ring-tailed lemurs in Madagascar.

Condition	Setting (abbreviation)	Setting description	Samples: collected (analyzed)	
			fecal	soil
wilderness	Amoron'I Onilahy (AMO)	Riverine gallery forest, dry scrub forest	20	4 (3)
	Berenty Reserve (BER)	Semi-arid dry forest, spiny forest	22 (19)	4
	Beza Mahafaly Special Reserve (BEZ)	Riverine gallery and semi-arid spiny forest	26	4 (3)
	Fiheranana (FIH)	Dry forest and spiny forest	2	-
	Isalo National Park (ISO)	Dry deciduous forest	18 (16)	3
	Ivohiboro (IVO)	Humid forest, grassland	15	-
	Ranomay (RAN)	Dry forest	13	2 (1)
	Tsimanampetsotsa National Park (TSI)	Dry forest and spiny forest	29 (28)	8
captivity: Madagascar	Lemur Rescue Center (Toliara, Madagascar; LRC)	Outdoor enclosures in dry and spiny forest	33	2 (1)
	Various towns (pets)	Pets housed in human dwellings	8	-
captivity: U.S.	Duke Lemur Center (Durham, NC; DLC)	Indoor and outdoor enclosures, including free ranging in semi-deciduous forest	22	3 (2)
	National Zoological Park (Washington, DC; NZP)	Indoor and outdoor enclosures, moated island with vegetation	4	-
	North Carolina Zoo (Asheboro, NC; NCZ)	Indoor and outdoor enclosures, moated island with vegetation	3	-
		Total samples:	215 (209)	30 (25)



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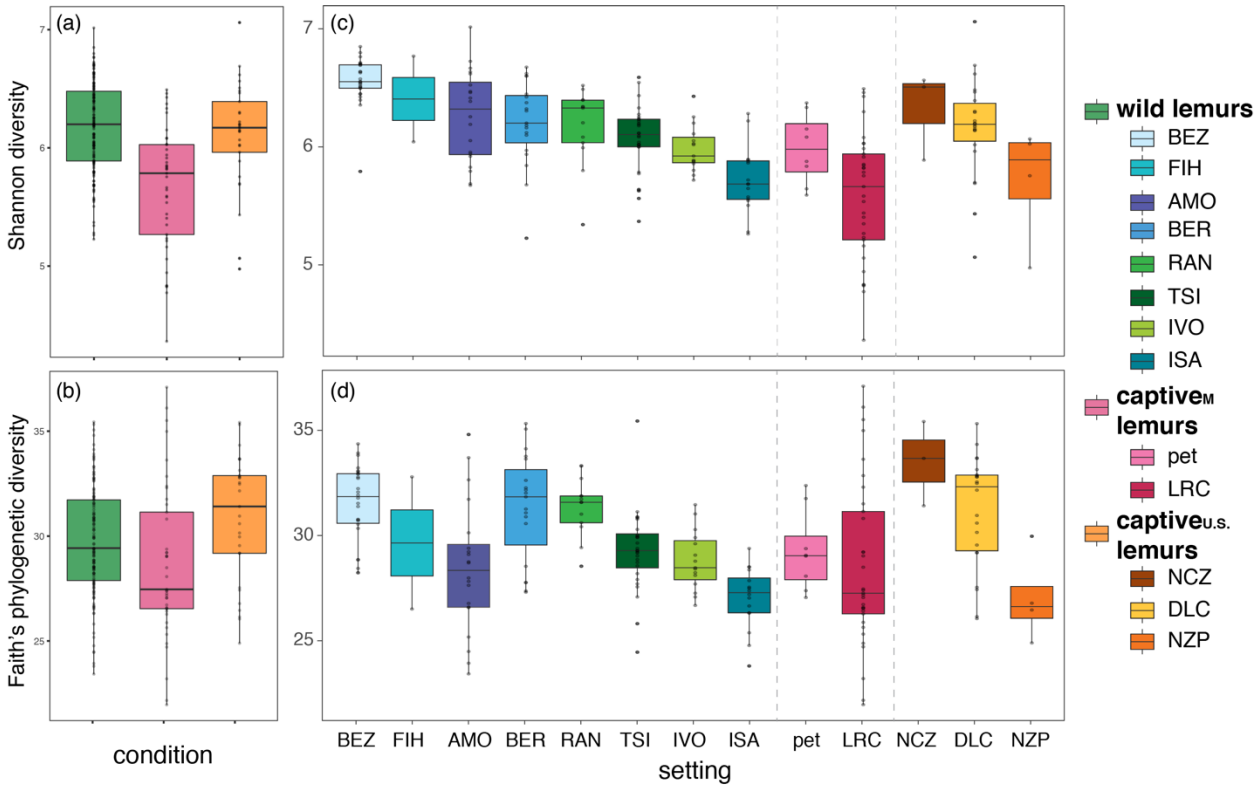

Figure 1. Alpha diversity metrics of lemur gut microbiota (a, b) collapsed by environmental condition, including wilderness (wild lemurs; green), captivity in Madagascar (captive$_M$ lemurs; pink), and captivity in the U.S. (captive$_{U.S.}$ lemurs; orange), and (c, d) averaged across individuals for each of the 13 different settings inhabited (reprising the color codes of each condition, delineated by dashed vertical lines). Shown are both (a, c) Shannon diversity and (b, d) Faith's phylogenetic diversity. Across the (c, d) settings within a condition (see Table 1 for names of abbreviated study sites), the data are plotted in descending order of mean Shannon diversity.

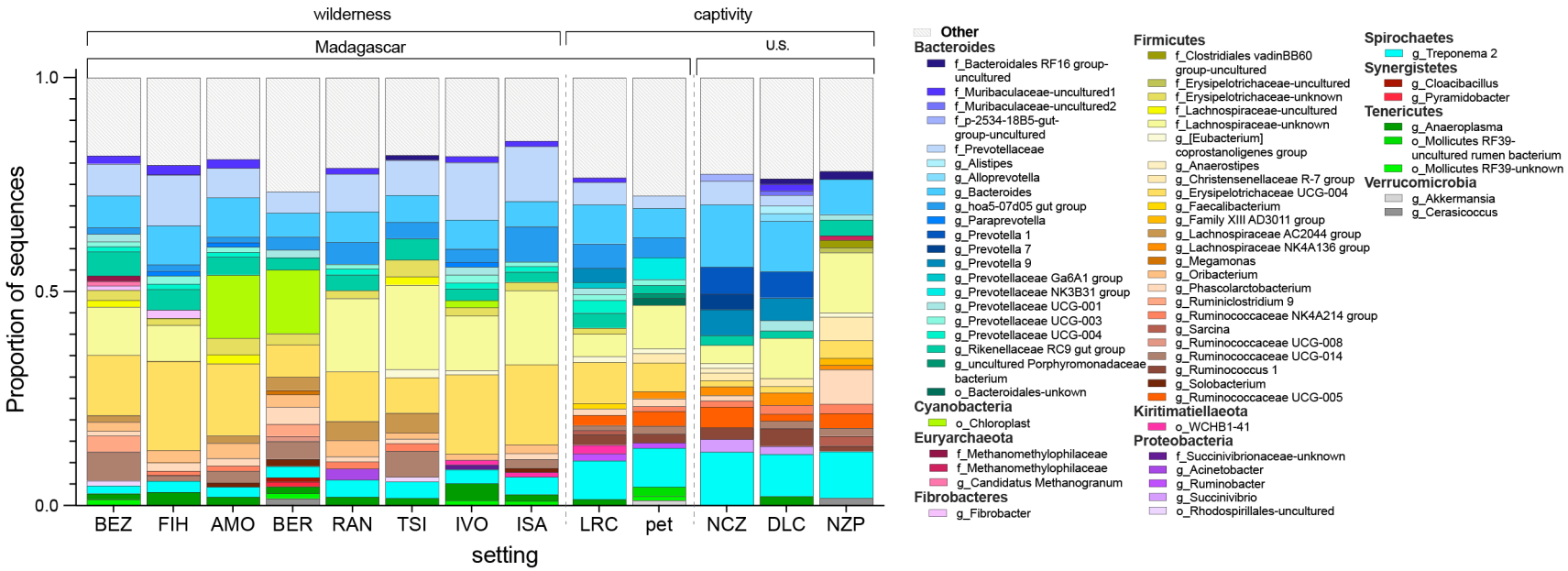

Figure 2. Mean proportion of sequences assigned to microbial taxa across lemurs at each of the 13 different settings, with the three conditions (wilderness, captivity in Madagascar, and captivity in the U.S.) delineated by dashed vertical lines (see Table 1 for names of abbreviated study sites). Taxa are identified by phylum and deepest possible taxonomic level (i.e., genus level or above); those representing < 1% of the microbiomes were combined into the category "Other"

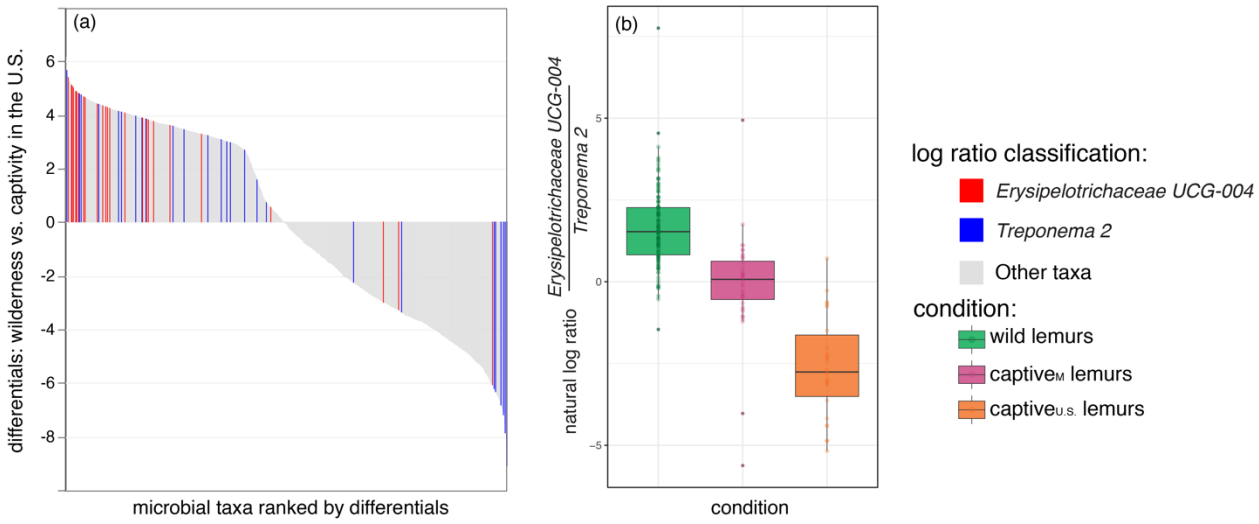

Figure 3. Differential abundance of *Erysipelotrichaceae UCG-004* and *Treponema* 2 in the gut microbiota of lemurs. (a) Differential rank plot showing lemur gut microbial taxa (x axis) ranked by their differentials (y axis; the estimated log-fold changes for taxa abundances across sample groups) for lemurs in the wilderness vs. captivity settings in the U.S. Those taxa that are more abundant in the wild lemurs compared to captive lemurs in the U.S. appear on the right side of the plot whereas those that are less abundant in wild lemurs appear on the left side. *Erysipelotrichaceae UCG-004* and *Treponema 2* differentials are highlighted in red and blue, respectively, with other taxa represented in gray. (b) Natural log ratios of *Erysipelotrichaceae UCG-004* vs. *Treponema 2* in lemurs across all three environmental conditions.

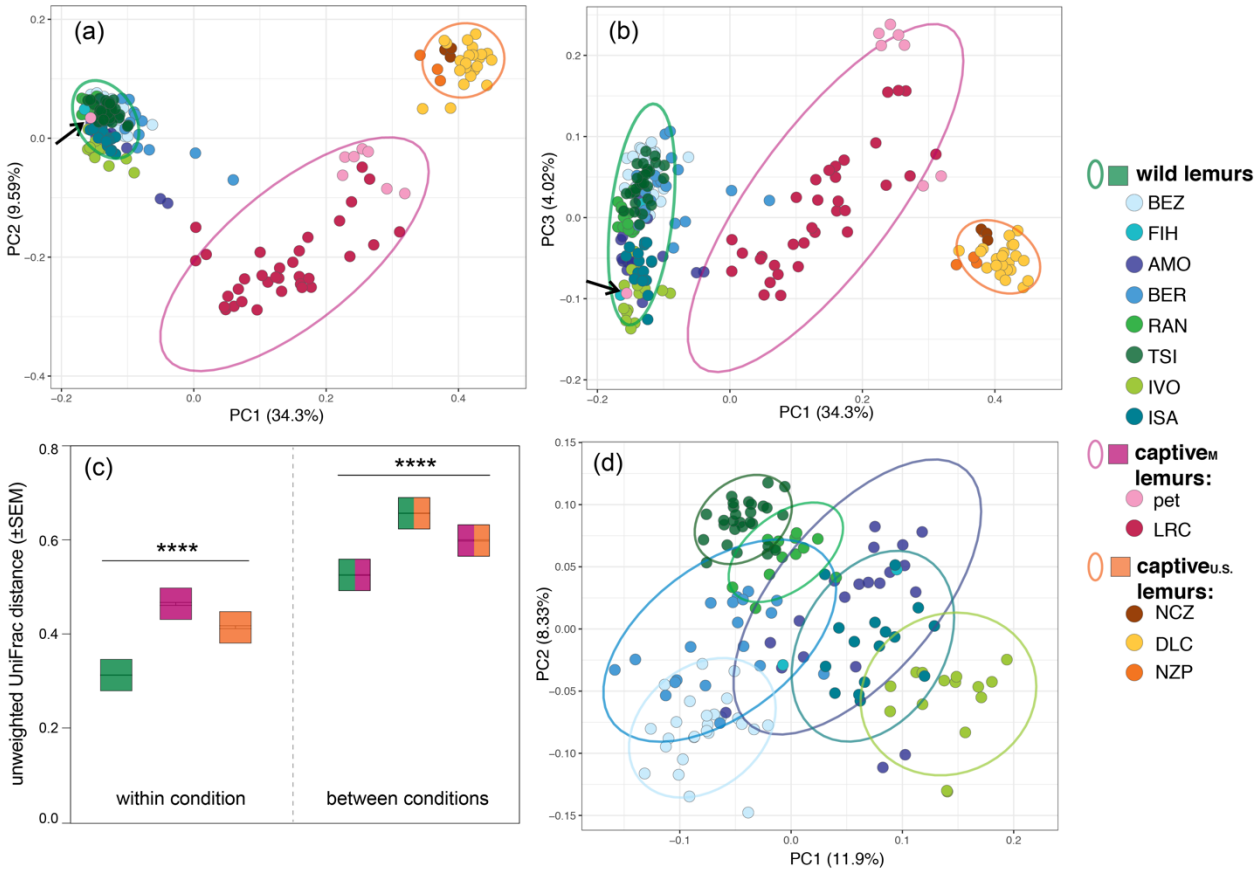

Figure 4. Beta diversity (unweighted UniFrac distances) of lemur gut microbiota across three environmental conditions – wilderness (wild lemurs; green), captivity in Madagascar (captive$_M$ lemurs; pink), and captivity in the U.S. (captive$_{U.S.}$ lemurs; orange) – that encompass 13 setting (see Table 1 for names of abbreviated study sites). (a, b) Principal coordinate plots, showing axes 1 and 2, or 1 and 3, respectively, of individual gut microbial communities colored by setting and encircled by normal data ellipses reflecting environmental condition. (c) Mean beta diversity distance scores within (single color) and between (two colors) environmental conditions. (d) Principal coordinate plots, showing axes 1 and 2, for the eight settings within the wilderness condition. Kruskal-Wallis test with Benjamini-Hochberg correction; **** $p < 0.0001$.

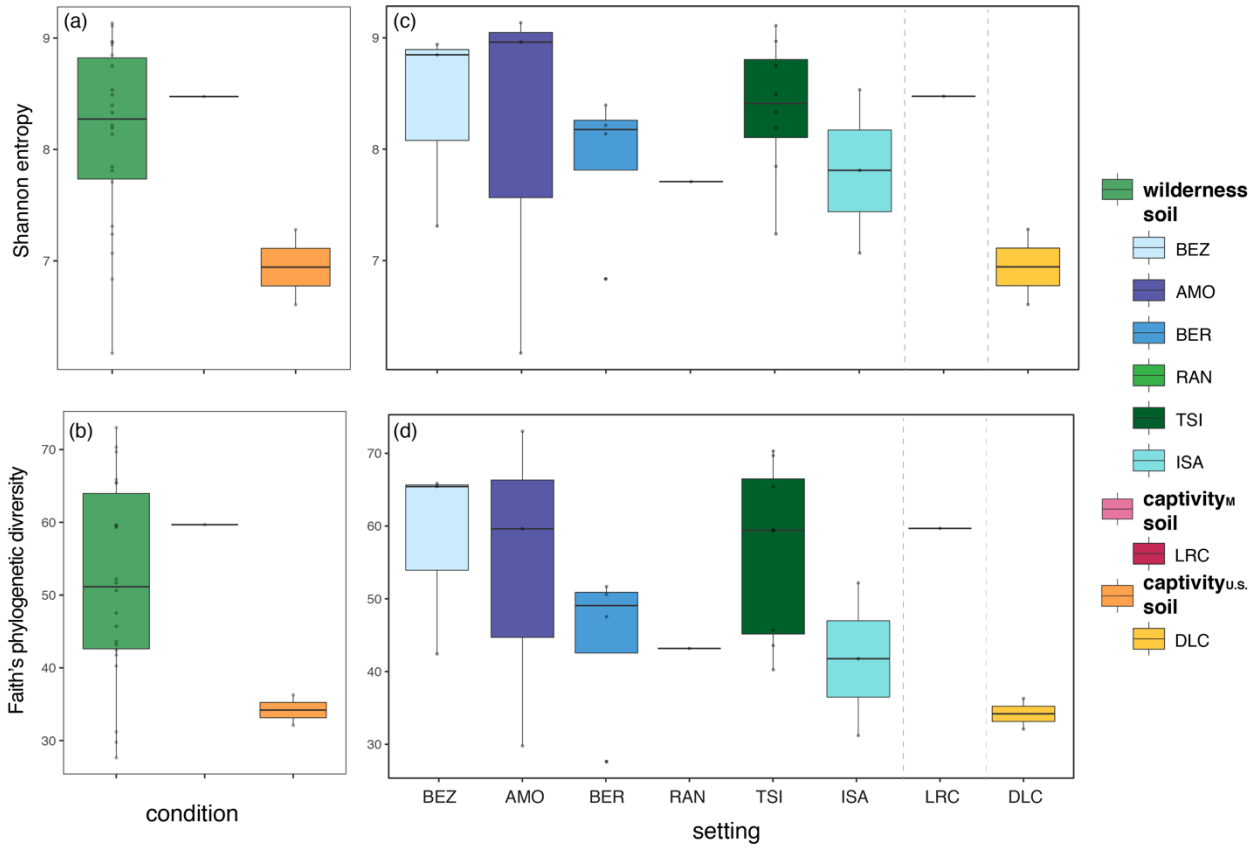

Figure 5. Alpha diversity metrics of soil microbiota (a, b) collapsed by environmental condition, including wilderness (wilderness soil; green), captivity in Madagascar (captivity$_{M}$ soil; pink), and captivity in the U.S. (captivity$_{U.S.}$ soil; orange) and (c, d) averaged across individuals for each of the eight different settings (reprising the color codes of each condition, delineated by dashed vertical lines). Shown are both (a, c) Shannon diversity and (b, d) Faith's phylogenetic diversity. Across the (c, d) settings within a condition (see Table 1 for names of abbreviated study sites), the data are plotted in descending order of mean Shannon diversity.

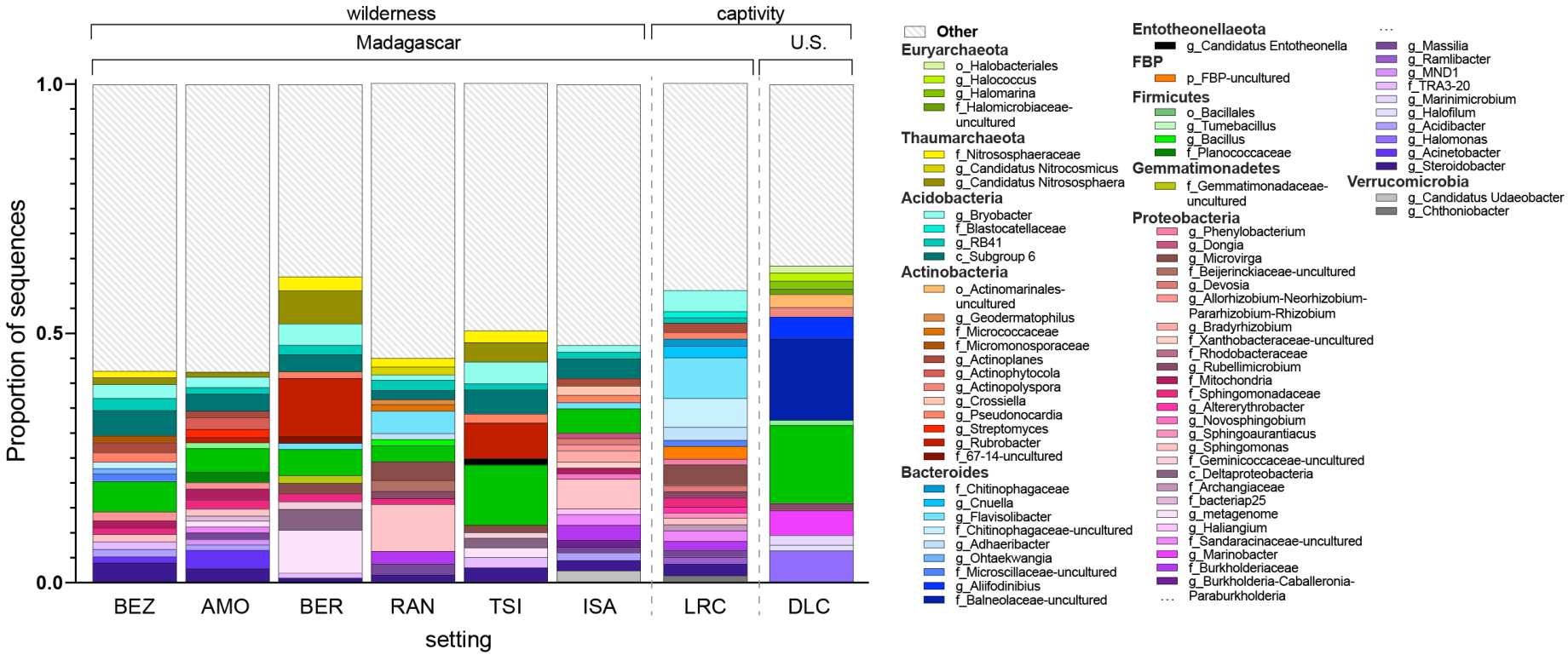

Figure 6. Mean proportion of sequences assigned to microbial taxa of soil at each of the eight settings sampled, within the three conditions: wilderness (wilderness soil; green), captivity in Madagascar ($captivity_M$ soil; pink), and captivity in the U.S. ($captivity_{U.S.}$ soil; orange), which are delineated by dashed vertical lines (see Table 1 for names of abbreviated study sites). Taxa are identified by phylum and deepest possible taxonomic level (i.e., genus level or above); those representing < 1% of the microbiomes were combined into the category "Other".

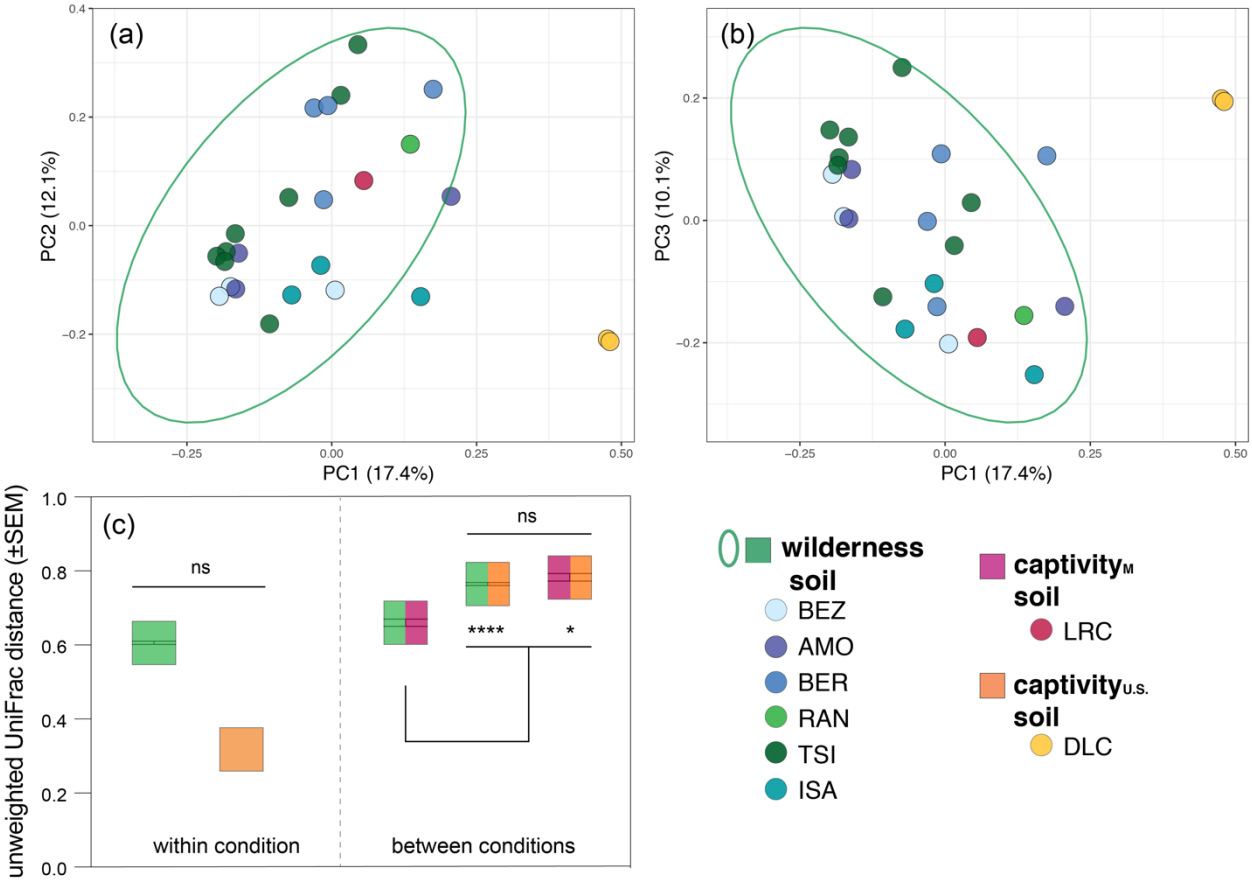

Figure 7. Beta diversity (unweighted UniFrac distances) of soil microbiota across three environmental conditions - wilderness (wilderness soil; green), captivity in Madagascar (captivity$_M$ soil; pink), and captivity in the U.S. (captivity$_{U.S.}$ soil; orange) – that encompass eight setting (see Table 1 for names of abbreviated study sites). (a, b) Principal coordinate plots, showing axes 1 and 2, or 1 and 3, respectively, of soil microbial communities colored by setting and encircled by normal data ellipses reflecting environmental condition. (c) Mean beta diversity distance scores within (single color) and between (two colors) environmental conditions. Kruskal-Wallis test with Benjamini-Hochberg correction; * $p < 0.05$, **** $p < 0.0001$.

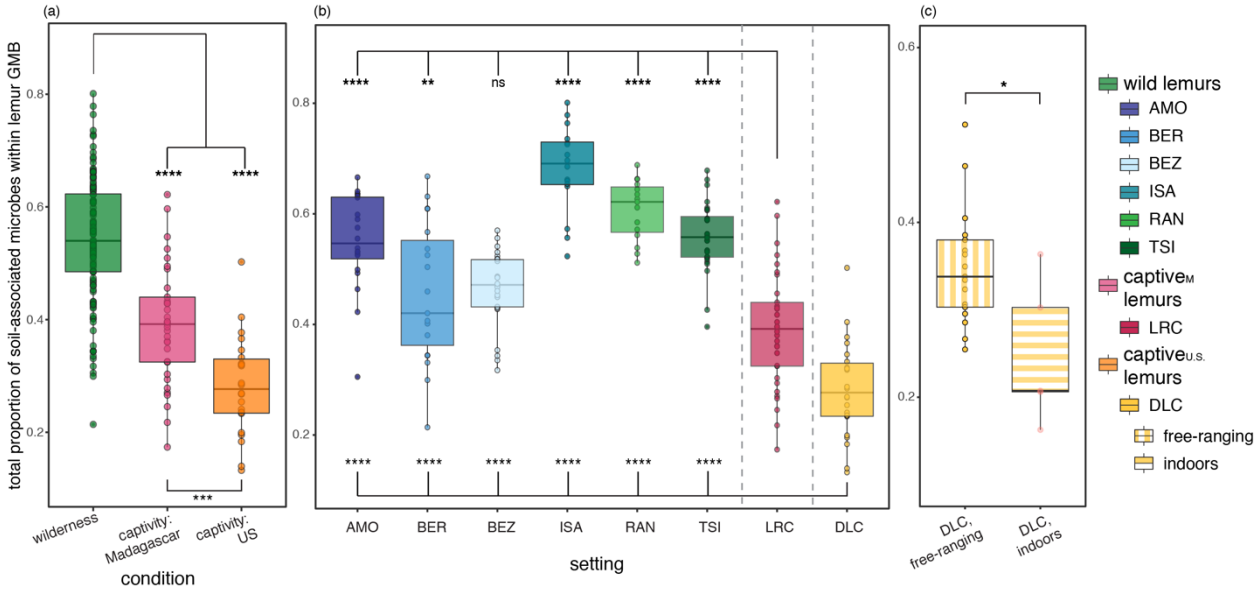

Figure 8. Mean proportion of total soil-associated microbes in the gut microbiota of lemurs (a) collapsed by environmental condition: wilderness (wild lemurs; green), captivity in Madagascar ($captive_M$ lemurs; pink), and captivity in the U.S. ($captive_{U.S.}$ lemurs; orange). Averaged soil-associated microbes across individuals for (b) each of the eight different settings (reprising the color codes of each condition, delineated by dashed vertical lines) and (c) at the Duke Lemur Center (DLC) that were semi-free-ranging in natural habitat enclosures or were housed indoors. Kruskal-Wallis test with pairwise comparisons and Benjamini-Hochberg correction; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, ns = nonsignificant.

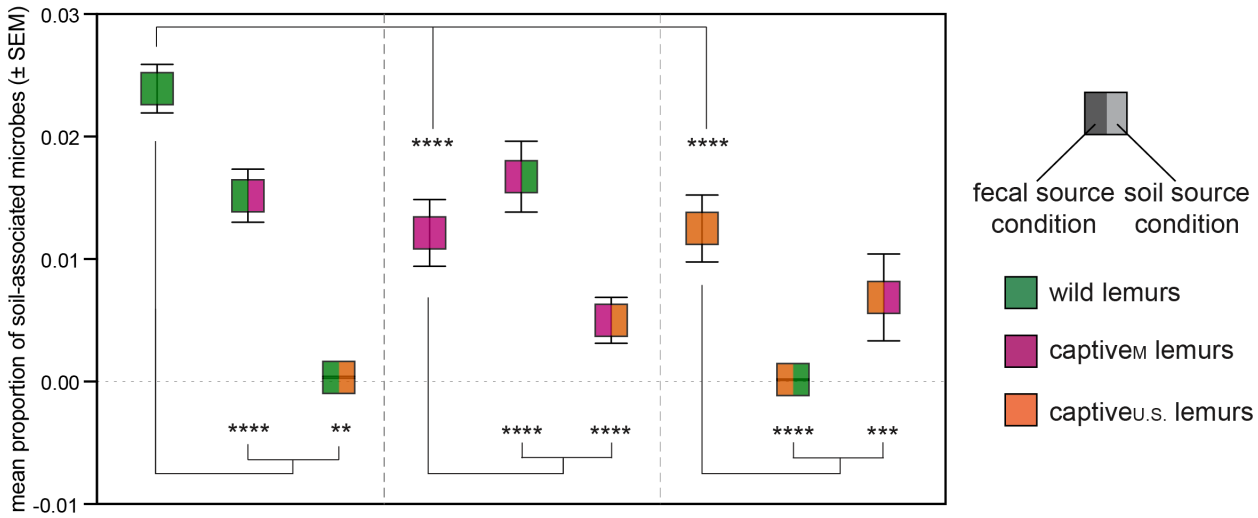

Figure 9. Mean proportion of soil-associated microbes in the gut microbiota of lemurs within (single color) and between (two colors) the three conditions: wilderness (wild lemurs; green), captivity in Madagascar ($captive_M$ lemurs; pink), and captivity in the U.S. ($captive_{U.S.}$ lemurs; orange) – that encompass eight setting. Within the gut microbiota of lemurs from a given condition (first color = fecal source condition), values show the proportion of soil associated microbes from a given condition (second color = soil source condition). Kruskal-Wallis test with pairwise comparisons and Benjamini-Hochberg correction; ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.