Title: Host phylogeny and host ecology structure the mammalian gut microbiota at different taxonomic scales

Running title: Gut microbiota of mammalian herbivores

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Abstract

The gut microbiota is critical for host function. Among mammals, host phylogenetic relatedness and diet are strong drivers of gut microbiota structure, but one factor may be more influential than the other. Here, we used 16S rRNA gene sequencing to determine the relative contributions of host phylogeny and host dietary guild in structuring the gut microbiotas of 11 herbivore species from 5 families living sympatrically in southwest Kenya. Herbivore species were classified as grazers, browsers, or mixed-feeders. We found that gut microbiotas were highly species-specific, and that host family accounted for more variation in the gut microbiota (35%) than did host dietary guild (14%). Overall, similarity among gut microbiotas increased with host phylogenetic relatedness (r=0.73), yet this relationship was not apparent among seven closely related Bovid host species (r=0.21 NS). In bovids, host dietary guild explained twice as much variation in the gut microbiota as did host species. Lastly, we found that the gut microbiotas of herbivores residing in southwest Kenya closely resemble those of conspecifics from central Kenya, suggesting that regardless of variability in host local habitat, hosts consistently provide microbes with similar niches for colonization. Overall, our findings suggest that host phylogeny may structure the gut microbiota at broad taxonomic scales, but that host ecology may be more influential in shaping the gut microbiotas of closely related host species.

Background

The mammalian gastrointestinal tract harbors dense populations of microbes, which collectively are termed the gut microbiota. Resident gut microbes are known to promote the digestive efficiency of their hosts by synthesizing vitamins, breaking down fiber, and supplementing the host with energy released from fermentation [1–4]. The gut microbiota also interacts with the host immune system, and may also modulate behavior [5–7]. Due to the critical importance of the gut microbiota for mammalian gastrointestinal functioning, recent research has focused on identifying factors that influence the composition, structure, and assembly of these bacterial communities. Across a wide range of studies conducted with both captive and wild mammal populations, physiological factors such as host sex, age, and disease state can explain variation in the gut microbiota [8–11]. Additionally, a range of environmental factors including host habitat, season, and geography are also associated with gut microbiota structure [12–15]. However, the two main drivers of mammalian gut microbiota composition are consistently host diet and host phylogenetic relatedness.

Across large taxonomic scales, the mammalian gut microbiota varies with host diet, such that omnivores, carnivores, and herbivores exhibit distinct gut microbiota profiles [16–18]. Furthermore, changes in host diet are usually accompanied by changes in the gut microbiota. For instance, in Western lowland gorillas (*Gorilla gorilla gorilla*), the gut microbiota shifts during drier periods of the year, when gorillas transition from eating fruits to eating more herbs and leaves [19]. More fine-scale differences in diet are also associated with variation in the gut microbiota. In giant pandas (*Ailuropoda melanoleuca*), consumption of different bamboo parts (shoot vs. stem vs. leaf) or different bamboo species is correlated with different gut microbiota compositions [20, 21]. Nonetheless, the gut microbiota is also structured by host phylogenetic relatedness, such that closely related host species often have more similar gut microbiotas than more distantly related host

species [22–26]. This congruence between host phylogenetic relatedness and gut microbiota similarity is termed "phylosymbiosis" [27–29]. Phylosymbiosis can arise when closely related host species provide potential colonizing microbes with similar ecological niches, which may or may not be due to host overlap in diet, habitat, physiology, and/or behavior.

If phylogenetic relatedness is a more important predictor of the gut microbiota than host ecology, then gut microbiota similarity should increase with increasing phylogenetic relatedness among hosts regardless of their habitat or dietary preferences. Phylosymbiosis should be evident among hosts that share habitats or diets, as well as among hosts that reside in different habitats and consume different diets. For example, in mice, voles, and shrews, gut microbiotas tend to be more similar among closely related host species, despite these animals occupying different habitats [30]. In populations of American pikas (*Ochotona princeps*) from different mountain ranges, a cladogram of gut microbiota similarity was congruent with a phylogeny of host genetic similarity [31]. Within folivorous primates that had overlapping diets, gut communities exhibited patterns consistent with phylosymbiosis [23]. Furthermore, phylosymbiosis has been observed among host species that live sympatrically. Recently, Kartzinel *et al.* analyzed the gut microbiotas of 33 species of sympatric herbivores from the Laikipia region in central Kenya, across multiple seasons and found that host phylogenetic relatedness strongly predicted gut microbiota composition ($r = 0.91$). Host phylogenetic relatedness was only weakly correlated with host diet ($r = 0.28$) [32], suggesting that convergence of gut microbiotas among closely related hosts was not due to shared host ecology or diet, but potentially to shared evolutionary history among hosts, which could include similar evolutionary histories and relationships between the hosts and their resident microbiota.

Here, we build upon this earlier work and use 16S rRNA gene sequencing to determine the relative influences of host phylogenetic relatedness and host diet in structuring the gut microbiota of 11 species of herbivores living sympatrically in the Masai Mara National Reserve (henceforth the Masai Mara) in southwestern Kenya. We survey the gut microbiotas of African buffalo, domestic cattle, Common eland, impala, Kirk's dik-dik, Thompson's gazelle, topi, Masai giraffe, common warthog, plains zebra, and African elephant. These species represent 5 mammalian families (Bovidae, Elephantidae, Equidae, Giraffidae, and Suidae) and three dietary guilds: grazers, browsers, and mixed-feeders. We determine whether patterns observed among Laikipia herbivores [32] are also observed among herbivores in the Masai Mara. We also compare the gut microbiotas of herbivores from the two geographic regions to determine the extent to which host geography and/or local habitat influence the gut microbiota, as these two regions differ in their altitude, vegetation, mammal species and densities, and degree of human disturbance [33–37]. The two regions are about ~ 290 km apart.

Specifically, our study objectives were to 1) characterize the gut microbiota composition of 11 species of Masai Mara herbivores, 2) determine the relative contributions and amount of variance in the gut microbiota explained by host family and host species compared to host dietary guild, 3) determine whether phylosymbiosis was observed among our 11 study species, and in a reduced sample of 7 closely related Bovid

136 species, and 4) compare the gut microbiotas of Masai Mara herbivores to those from
137 conspecifics in Laikipia to evaluate whether phylosymbiosis is observed despite hosts
138 occupying different geographic regions and different habitat types. Collectively, our
139 findings elucidate the factors shaping the gut microbiota of hosts at greater and lesser
140 taxonomic scales, which has implications for host gastrointestinal functioning.
141
142 **Results**
143 ***Gut microbiota composition in 11 species of African herbivores.***
144 The gut microbiotas of the surveyed savanna herbivores (Table 1) were dominated by two
145 bacterial phyla, *Firmicutes* (51% average relative abundance across samples), and
146 *Bacteroidetes* (32% average relative abundance), and also contained significant
147 proportions of *Spirochaetes* (3%), *Verrumicrobia* (3%), *Proteobacteria* (2.4%), and
148 *Tenericutes* (1.8%) (Figure S2). The most abundant bacterial families across host species
149 were *Ruminococcaceae* (30.8% average relative abundance), *Rikenellaceae* (11.4%),
150 *Lachnospiraceae* (10.9%), and *Prevotellaceae* (8%). The gut microbiotas also contained
151 unclassified *Bacteroidales* (3.9%), and bacteria from the *Bacteroidaceae* (3.7%),
152 *Spirochaetaceae* (3.7%), *Akkermansiaceae* (2.9%), and *Christensenellaceae* (2.8%) families
153 (Figure 1A). Prevalent bacterial genera included *Alistipes*, *Bacteroides*, *Ruminococcus*, and
154 *Treponema* (Figure S3).
155
156 A total of 10 Amplicon Sequence Variants (ASVs) were present in >90% of the
157 samples and represented core bacterial taxa; 7 were assigned to the family
158 *Ruminococcaceae*, 1 to *Peptococcaceae*, and 2 to *Lachnospiraceae* (*Agathobacter*).
159 Differences in gut microbiota composition between host species were also evident (Figure
160 1A). The gut microbiota of each host species contained ASVs that were widespread (i.e.
161 present in >75% samples) among that host species' samples yet were rarely present (i.e.
162 present in <3% samples) elsewhere; we refer to these ASVs as being unique to a particular
163 host species. Buffalo, cattle, topi, and impala gut microbiotas were mostly comprised of
164 ASVs that were present in other herbivores, as <2% of their ASVs were unique to each host
165 species. Between 4% and 8% of ASVs comprising the gut microbiota of dik-diks, eland,
166 elephant, Thompson's gazelle, and giraffe were unique to each particular host species.
167 Warthogs and zebras however, harbored distinct microbiotas, as 70-77% of their ASVs
168 were not present in the guts of the other African mammals.
169
170 ***Gut microbiota alpha-diversity varies with host family and dietary guild.***
171 Across our surveyed host taxa, gut microbiota richness, evenness, and phylogenetic
172 diversity varied with host family and dietary guild, but not with rainfall or average
173 maximum temperature over the prior two weeks of sampling (Table 2, Figure 2A). The
174 average minimum temperature over the prior two weeks was significantly associated with
175 gut microbiota phylogenetic diversity but not with richness or evenness (Table 2).
176 Specifically, lower minimum temperatures were associated with lower gut microbiota
177 phylogenetic diversity. Surprisingly, host fermentation type explained none of the observed
178 variation in the α-diversity of gut microbiotas (Table 2).
179
180 Post-hoc comparisons revealed that hosts from the Bovid and Equid families tended
181 to have richer gut bacterial communities than the other surveyed savanna herbivores,

whereas hosts from the Suidae and Elephantidae families generally harbored less diverse gut communities (Figure 2A; see Table S1 for exact post-hoc comparison values). Furthermore, grazers tended to have more diverse gut microbiotas than browsers (Linear Mixed Model (LMM) Chao 1 β=359.2 ±125.5 $p<0.01$, Shannon diversity β=0.36 ±0.17 $p<0.01$, Phylogenetic diversity β=-4.54 ±3.12 $p=0.19$) or mixed-feeders (LMM Chao 1 β=-466±138.4 $p<0.001$, Shannon diversity β=0.59 ±0.19 $p<0.01$, Phylogenetic diversity β=-4.45±3.44 $p=0.19$). The gut microbiota of mixed-feeders was more phylogenetically diverse than that of browsers (LMM Phylogenetic diversity β=-9 ±3.38 $p=0.023$), but the two groups harbored communities of similar richness and evenness (LMM Chao 1 β=-106.8 ±135.7, Shannon diversity β=0.22±0.18; all $p>0.05$) (Figure 2A).

Lastly, within a single host family (i.e. Bovidae), microbiota alpha-diversity varied with host species, dietary guild, and average minimum temperatures over two weeks (Figure 2C, Table 2). Specifically, greater higher minimum temperatures during the prior two weeks were associated with reduced gut microbiota diversity. Post-hoc comparisons revealed that, generally, cattle and impala harbored more diverse microbiotas than did buffalo or dik-diks (see Table S2 for exact post-hoc comparison values). Additionally, the gut microbiotas of browsers were less diverse than those of grazers (LMM Chao 1 β=-511.4 ±115.1 $p<0.0001$; Shannon diversity β=-0.77 ±0.15 $p<0.0001$; Phylogenetic diversity β=-8.81 ±3.02 $p=0.01$) or mixed-feeders (LMM Chao 1 β=-296.8 ±126.0 $p=0.02$; Shannon diversity β=-0.64 ±0.16 $p<0.0001$; Phylogenetic diversity β=-5.33 ±3.12 $p=0.13$) (Figure 2C). Gut microbiota diversity did not differ between grazers and mixed feeders (Chao 1 β=214.7 ±133.4, Shannon diversity β=-0.13 ±0.17; Phylogenetic diversity β=-3.48 ±3.26 $p=0.023$; all $p>0.05$).

Host taxonomy outweigh host dietary guild in structuring the gut microbiota.

The strongest predictors of gut microbiota structure across the 11 species of African herbivores were host family, followed by host dietary guild, and host species. These factors explained, on average, 24.74%, 13.54%, and 10.42% of the variation in the gut microbiota, respectively (Permutational Multivariate Analysis of Variance (PERMANOVA) analyses, Table 3). Sample month explained only 1.5% of the variation, and rainfall and temperature values during the prior two weeks were not predictive of gut microbiota composition (Table 3). Regardless of whether distance matrices took into account the proportions of bacterial taxa, their presence/absence, or their phylogenetic relatedness, the percent variation explained by each host factor was consistent. Thus, we only present PCoA ordination plots using the Bray-Curtis index for brevity. These plots show that the gut microbiota primarily partitions by host family, and within a family, the gut microbiota secondarily groups by host dietary guild (Figure 2B).

Within Bovidae, host dietary guild was a slightly stronger predictor of the gut microbiota (21.12% of the variation) than host species (17.19% of the variation) (Bray-Curtis, PERMANOVA) (Figure 2D) (Table 3). When taking into account the phylogenetic diversity of bacterial taxa in the gut microbiota (weighted Unifrac metric, PERMANOVA), host dietary guild explained twice the amount of variation (21.74%) as that accounted for by host species (10.47%) (Table 3). PCoA ordinations showed that the gut microbiotas of each dietary guild formed distinct clusters, but were also differentiated by host species

(Figure 2D). Indeed, when controlling for host dietary guild, the microbiota is highly-species specific and host species accounts for 49% of the observed variation (PERMANOVA: Bray-Curtis R^2=0.48; Jaccard R^2=0.44; Weighted Unifrac R^2=0.49, Unweighted Unifrac R^2=0.47; all p=0.001) (Figure S4).

Bacterial taxa are enriched in particular herbivore hosts.

Indicator species analysis showed that the gut microbiotas of elephants maintained higher proportions of *Endomicrobiaceae* and *Desulfobulbaceae*, Zebras of *Helicobacteraceae* and *Deltaproteobacteria*, and warthogs of *Myxococcales* and *Coxiellaceae*. Giraffe gut microbiotas were enriched in *Enterobacteriaceae*, *Bifidobacteriaceae*, and *Bacillaceae* (Figure 1B).

Similarly, the gut microbiotas of hosts from different dietary guilds were enriched in specific types of bacteria. Grazer gut microbiotas contained greater abundances of *Sphingobacteriaceae*, *Flavobacteriaceae*, *Neisseriaceae*, and *Lentisphaeria* (Figure 1C) than those of browsers or mixed feeders. Browser gut microbiotas were enriched in 11 bacterial taxa, including *Bacillaceae*, *Coriobacteriales, Methanomicrobia* and *Rubrobacteriaceae* (Figure 1C). Lastly, the gut microbiotas of mixed feeders harbored high proportions of *Synergistaceae, Succinivibrionaceae*, and *Bacteroidales*, among other bacteria (Figure 1C).

Evidence of phylosymbiosis in the gut microbiotas of African herbivores.

Our results indicate that gut microbiota similarity increased with host phylogenetic relatedness (mantel test Bray-Curtis r=0.76, p=0.006; Jaccard r=0.72, p=0.006; Weighted Unifrac r=0.75, p=0.007; Unweighted Unifrac r=0.72, p=0.007). The gut microbiota tended to be more similar among closely related host taxa (e.g. buffalo and cattle) than among distantly related host taxa (e.g. impala and elephant) (Figure 3A & Figure 3B). Despite warthogs being more closely related to bovids than to elephants and zebras (Figure 3A), they had highly dissimilar communities from all of the herbivore species examined, and pairwise comparisons that include this host species deviate from the trendline (Figure 3B). Importantly, among closely related species in the Bovid family, we found no significant relationship between host phylogenetic relatedness and gut microbiota similarity (mantel test Bray-Curtis r=0.27, p=0.09; Jaccard r=0.20, p=0.08; Weighted Unifrac r=0.22, p=0.059; Unweighted Unifrac r=0.16, p=0.08).

Host geographic region does not strongly predict the gut microbiotas of Masai Mara and Laikipia herbivores.

We compared the gut microbiota structure of eight herbivore species (African buffalo, domestic cattle, common eland, impala, giraffe, plains zebra, common warthog, and African elephant) inhabiting the Masai Mara (this study) and the Laikipia region (Kartzinel *et al.* [32]) in Kenya. We found that host geographic region explained little of the observed variation in the gut microbiota (<2%, with the exception of weighted Unifrac distances, where it explained 6%) (Table S3). The gut microbiotas were primarily structured by host species and host dietary guild, which explained on average, 39% and 12% of the variation, respectively (Figure 4A, Table S3). Sample month explained an additional 2-3% of the variation (Table S3).

When examining each host species separately, host geographic region weakly predicted gut microbiota structure (Table S3). Here, variation in sample month was more strongly associated with variation in the gut microbiota (accounting for up to 7% of the variation) than was host geographic region (which accounted for 1-6% of the variation). Furthermore, within each host dietary guild, the gut microbiota clustered closely by host species and this factor explained >50% of the observed variation, despite hosts residing in two geographic regions (Table S3). Lastly, patterns consistent with phylosymbiosis were also observed in herbivores from both geographic regions in Kenya. Gut microbiota similarity increased with host phylogenetic relatedness (mantel test Bray-Curtis r=0.67, p=0.016; Jaccard r=0.76, p=0.005; Weighted Unifrac r=0.62, p=0.011; Unweighted Unifrac r=0.68, p=0.027). Similar to what was observed earlier in our Masa Mara study animals, phylosymbiosis was not evident among the four species of bovids included in both studies (mantel test Bray-Curtis r=0.92, p=0.08; Jaccard r=0.92, p=0.08; Weighted Unifrac r=0.83, p=0.16; Unweighted Unifrac r=0.92, p=0.08).

When comparing gut microbiota compositions between the herbivores of the two regions, we found that each species of herbivore contains similar relative abundances of the predominant bacterial ASVs as those found in their conspecifics in the other region (Figure 4B). Only elephant and warthog gut microbiotas appear to differ between hosts in the Masai Mara and Laikipia regions.

Discussion

Evidence of phylosymbiosis in sympatric African herbivores.

Our results showed that phylosymbiosis was observed among 11 species of herbivores living sympatrically in the Masai Mara . Patterns of phylosymbiosis have been documented extensively across vertebrate groups, including primates, rodents, ruminants, carnivores, reptiles, and insects [23–26, 32, 38, 39]. However, in many of these studies, host species do not occur sympatrically, and usually inhabit multiple habitats across the globe. Nonetheless, in addition to findings documented for herbivores in the Laikipia and Masai Mara regions of Kenya, evidence of phylosymbiosis among host species living in sympatry has been observed in seven species of deer mice [40], six species of Malagasy mammals [41], twelve species of lemurs [42], and nine species of diurnal, non-human primates [43].

The mechanisms and processes that yield patterns of phylosymbiosis have not yet been elucidated, but host ecological and phenotypic traits are likely acting as filters and thus shaping microbial community assembly. Closely related hosts are potentially facilitating colonization by the same microbial types, due to similarities in their morphology, anatomy, digestive physiologies, and immune system components [16, 44, 45]. Specifically, related hosts may possess similar antimicrobial peptides and toll-like receptors that serve to filter similar bacterial clades from the environment [46, 47]. Closely related hosts may further develop immune tolerance via adaptive immunity to the same symbiotic, commensal, and transient microbes [46, 47]. Lastly, some phylogenetically related hosts may also possess similar social group structures and pathways for transmitting microbes among group-mates, thereby contributing to patterns of phylosymbiosis. Overall, accumulation of differences in host traits as hosts diverged from

one another could potentially provide enough habitat differentiation in the gut to promote the divergence of symbiotic bacterial communities.

Furthermore, we found that phylosymbiosis was also present in conspecific African herbivores living in allopatry, in the Masai Mara or Laikipia regions of Kenya, although the strength of the phylosymbiotic signal was slightly reduced compared to that observed for either sympatric population considered in isolation. Overlap in gut microbiota structure is thought to be lower in allopatric animal populations than in sympatric animals due to variation introduced by habitat, dietary differences, and the spatial limits of bacterial dispersal [26], which could lead to slight differences in gut microbiota compositions among conspecifics inhabiting different geographical areas.

Host taxonomy outweighs influences of host ecology in structuring the gut microbiota across herbivore species.

Across the surveyed herbivores, host family and species were the strongest predictors of gut microbiota structure followed by host dietary guild. Gut microbiota clustering by host species is widespread, and is commonly reported in the majority of comparative gut microbiome studies. Host species may vary in their body size, behavior, neuroendocrine system, immune system, and metabolism, any of which could potentially shape the compositions of their gut microbiotas [16, 45, 48, 49].

Additionally, our study found that conspecific hosts from different geographic regions (Masai Mara, Kenya vs. Laikipia, Kenya) did not necessarily possess different gut microbiotas. Despite differences between the two regions in their climate, soil geochemistry, plant communities, and resident herbivore species, and potentially in their bacterial species pools, hosts are being colonized by the same bacterial types. The gut microbiotas of herbivores from Laikipia and the Masai Mara were inhabited by bacteria that, not only were likely performing very similar metabolic functions, but were the same sequence variants. Hosts from the two geographic regions are evidently filtering the same bacterial clades from the environment and are providing microbes with similar niches for colonization, leading to similar gut microbiota profiles. These findings suggest that there is strong selection for hosts to associate with particular microbial symbionts and for microbes to associate with particular animal hosts [47, 50]. Hosts have evolved sophisticated adaptations to live in symbiosis with a dense and diverse gut microbiota and bacteria have also evolved adaptations to reside in their host's body niches [47, 50]. In many vertebrates, microbes have enabled their hosts to adapt to changes in their environment and access new food resources, niches, or metabolites, thereby fostering close associations between hosts and their gut microbes [50, 51].

Within a host family, among closely related hosts, host ecology is a stronger predictor of the gut microbiota than across host families.

Within a group of closely related Bovid species in the Masai Mara, variation in the gut microbiota was more strongly associated with host ecology than host phylogeny. Gut microbiota similarity did not increase with host phylogenetic relatedness in bovids, and host dietary guild explained twice as much variation as did host species in the bovid data set. In other words, here between-species variation was less than the variation introduced

by differing diets. Additionally, gut microbiota alpha-diversity varied with environmental variables like minimum temperature during the two weeks before sampling, when this variable was not significant in the overall dataset.

Similarly, other studies report that local habitat, and not phylogenetic relatedness, predicts the structure of the gut microbiota among closely related hosts. For example, in lemurs (*Eulemur* spp., *Propithecus* spp.), phylosymbiosis was observed across but not within two host lineages, and within host lineages, host habitat (dry forest vs. rainforest) was significantly correlated with gut microbiota diversity [42]. In six species of chipmunk (*Tamias* spp.), gut microbiotas primarily clustered by host geography rather than by host species [52]. Lastly, in populations of yellow (*Papio cynecephalus*) and anubis baboons (*Papio anubis*), gut microbiota dissimilarity did not increase with host genetic distance, but did vary with their habitat's soil chemistry [53]. Because the bovids surveyed here are closely related, their bacterial communities are already very similar, and variation in their gut microbiotas likely result from fine-scale differences in diet, and variation in the local environment. While all the bovids are herbivores and ruminants, they do consume different plant parts (grass vs. shrubs vs. trees) [17, 54–56], and the gut microbiota can reflect these differences [20, 21]. Lastly, the gut microbiota is dynamic and highly responsive to changes in its hosts internal and external environment, which is why gut microbiota variation was correlated with rainfall and temperature in bovid hosts.

High gut microbiota alpha-diversity in grazing herbivores.
Results showed that gut microbiota alpha-diversity was considerably higher in grazers than in browsers or mixed-feeders. While mixed-feeders, including the omnivorous warthog, may have greater dietary breadths than grazers or browsers, they did not have more diverse gut communities. This is in accordance with prior findings, which report that the most diverse host diets do not always correlate with the most diverse microbiotas [32, 57, 58]. However, group size has been shown to correlate with gut microbiota diversity [59, 60], and the grazers in our study (e.g. buffalo, topi, zebras) forage in large herds, compared to the two browser species (giraffes, dik-diks) or some of the mixed-feeders (e.g. warthogs), which live in smaller groups. Frequent social interactions and interactions with a greater number of individuals is known to promote species richness in individual gut microbiotas [60, 61].

Additionally, when comparing the diversity of gut microbiotas among host families, bovids and equids harbored more diverse gut communities than hosts from *Suidae* or *Elephantidae*. Zebras in particular may have high alpha diversity because they consume the tops of grasses, which are lower in quality and contain a higher proportion of cellulose and lignin compared to young shoots, which is the preferred food source for some of the Bovid hosts [62]. Digestion of this tough plant material may require the enzymes and metabolisms from a diversity of bacterial types.

Gut microbiota composition reflects host dietary requirements in herbivores.
Across our surveyed herbivores, the most abundant bacterial taxa in the gut microbiota were *Ruminococcaceae*, *Rikenellaceae, Lachnospiraceae,* and *Prevotellaceae* which represent core taxa previously found in the gut microbiotas of many ruminants and

herbivores in general, including cervids and bovids [22, 63], equids [64], elephants[65], and giraffes[66]. *Ruminococcaceae* and *Lachnospiraceae* have also been found in the guts of folivorous primates [3] and in domestic pigs [67, 68]. Members of these bacterial families are responsible for digesting the cellulose, hemicellulose, lignin, and protein found in leaves, bark and grass, and fermenting these into short-chain fatty acids (SCFAs) such as acetate, succinate, butyrate, propionate [69] . These SCFAs are usable forms of energy for their hosts [70] and they can further contribute to host colonocyte growth, immune defense, and anti-inflammatory responses [1]. These bacterial taxa also possess fiber-degrading capabilities and can provide their hosts with protection against ingested toxic plant secondary metabolites [71].

Not all bacteria in the gut are directly contributing to their host's physiology; some may be commensals that cross-feed with keystone bacteria [72], while others may protect their hosts from pathogens through competitive exclusion or the synthesis of antimicrobial peptides [73, 74]. From an evolutionary perspective, it can be advantageous for hosts to harbor complex gut communities so that they can respond to novel environmental conditions and recover from disturbances (e.g. antibiotics or infection).

Conclusions

Our study showed that phylosymbiosis was observed in 11 species of sympatric herbivores residing in the Masai Mara, Kenya. Additionally, we found that the gut microbiota exhibited high species-specificity. The gut microbiotas of eight species of herbivores residing in two geographic areas in Kenya (Masai Mara and Laikipia) were very similar between conspecifics, and contained the same bacterial types. Overall, these findings suggest strong associations between herbivore hosts and their gut microbes, as well as the likelihood that related hosts are providing microbes with similar niches for colonization. Future studies should examine whether phylosymbiosis is present in the gut metagenomes of African herbivore species, and whether archaeal or fungal communities vary with host species and are related to host phylogeny.

Methods

Study location and sampling.

Fecal samples (N=181) were collected opportunistically from 11 species of herbivores permanently residing in the Talek and Mara Triangle regions of the Masai Mara (1°22′19″S, 34° 56′17″E) from March-June 2018 (Table 1). This Reserve is covered by open rolling grassland interspersed with seasonal watercourses and riparian bushes and trees. It has two rainy seasons (March-May and November-December, with annual rainfall >1000mm), and most of our sampling took place during the rainy months. Although the Masai Mara is home to small resident populations of zebra and wildebeest, millions of these individuals migrate into the reserve from July-October every year. As sampling occurred here before July, samples from wildebeest and zebras were limited. This is in contrast to Laikipia, which is more arid, and rainfall is "trimodal", averaging 300-600 mm annually [34, 36]. Elephant, impala and dik-dik are the dominant large herbivores in this region [75].

For fecal sample collection, we either observed animals defecating or identified species-of-origin based on the size, shape, and consistency of fresh dung, following

Kartzinel *et al*. (2019). Samples were then placed in sterile cryogenic vials and stored in liquid nitrogen until they were transported to Michigan State University, where they remained frozen at -80°C until nucleic acid extraction. For a list of all samples and their associated metadata, see the Github repository for this project (https://github.com/rojascon/Rojas_et_al_2020_African_herbivores_gut_microbiome).

While we did not directly collect diet data from the surveyed herbivores, we used Kingdon's *East African Mammals* [76–79] to classify our study species into their respective dietary guilds. Information about animal's fermentation type (e.g. foregut vs hindgut) was obtained from previously published sources [17, 54–56].

DNA extraction and 16S rRNA gene sequencing.

Fecal samples were sent to the University of Chicago at Illinois (UIC) Sequencing Core for automated DNA extractions using QIAGEN DNeasy PowerSoil kits (Valencia, CA, USA). DNA Concentrations of the fecal sample extracts were quantified using Qubit. The V4 region of the 16S rRNA gene was targeted for sequencing on the Illumina MiSeq platform at the Michigan State University Genomics Core, using published protocols by Caporaso *et al.* 2012 [80] and Kozich *et al.* 2013 [81].

Sequence processing and bioinformatics.

Sequences were processed in R (v.3.6.2) [82] using the Divisive Amplicon Denoising Algorithm (DADA2) pipeline (v1.14.1) [83] to infer amplicon sequence variants (ASVs). Briefly, reads were filtered for quality, allowing for 2 and 3 errors per forward and reverse read, respectively (trimLeft = c(10, 10), maxN = 0, maxEE = 2, truncQ = 2). Forward reads were trimmed to 240bp and reverse reads to 200bp; these paired-end reads were merged. Sequences were then dereplicated to remove redundancy and ASVs were inferred by pooling reads from all samples. Prior to creating the ASV abundance table, chimeras were removed and ASVs were taxonomically classified using the SILVA rRNA gene reference database (v.132) [84] with an 80% confidence threshold. ASVs taxonomically assigned as Eukarya, Chloroplasts, or Mitochondria, were removed from the dataset, as were those of unknown Kingdom origin; 12,938 total ASVs remained. The resulting ASV table and the taxonomic designations of the ASVs are available on GitHub. On average, samples retained over 70% (± 11%) of their total sequences after processing in DADA2. Nineteen samples did not amplify well (<400 sequences after processing) and were removed from the dataset. Most of these samples belonged to browser species (giraffe and dik-dik) suggesting that there may have been PCR inhibitors in their fecal samples (e.g. humic acid, tannins) that prevented successful extraction of DNA or library preparation. Table 1 has the sample sizes (N) for each study species before and after this filtering.

Composition and α-diversity statistical analyses.

Statistical analyses and data visualization were completed in R unless otherwise stated. To visualize microbiota composition, stacked barplots were constructed in ggplot2[85]. These plots showed the bacterial phyla, families, and genera with average relative abundances greater than 1% across samples. We also identified core gut microbial taxa, here defined as ASVs present in >90% of samples across all host species. Furthermore, ASVs were identified as being unique to a particular host species if they were present in over 75% of

the samples from that host species but were also found in <3% of the samples from the other host species.

Prior to alpha-diversity analyses, we controlled for potential influences of sequencing depth by subsampling all samples to 17,000 sequences using the mothur (v.1.42.3) [86] sub.sample command. Four fecal samples did not meet this sequence cutoff criterion, and were excluded from all alpha-diversity analyses. Mothur was used to construct rarefaction curves of ASV richness vs. sequencing depth (Figure S1) and Good's coverage values averaged 97.78 ± 0.91 across all samples, indicating that sample coverage was high and appropriate for characterizing fecal microbiota profiles. These values are comparable to those typically reported for other mammalian gut microbiota studies [21, 22, 87].

Microbiota alpha-diversity was estimated using observed richness (number of ASVs), Chao1 Richness, and Shannon diversity calculated using the phyloseq package (v.1.33.0) [88]. To obtain measures of Faith's Phylogenetic Diversity (PD), we constructed a phylogenetic tree of ASV sequences using phangorn (v. 2.5.5) [89] and calculated PD using the picante package (v1.8.1) [90]. The effects of predictor variables on each measure of alpha-diversity across all samples were evaluated via linear mixed models using the lme4 package (v.1.1.23) [91], specifying host family, dietary guild, fermentation type, and average rainfall and temperature over two weeks as fixed variables and sample month as a random variable. A similar model was also built for bovid samples only, and included host species as a predictor in lieu of host family but excluded fermentation type, as all bovids are foregut fermenters. The significance of each predictor variable was determined by running Wald Chi. Sq ANOVA tests (α=0.05) on the full models using the car package (v.3.0.7) [92]. These tests were followed by TukeyHSD post-hoc tests with Benjamini-Hochberg adjustments to control for multiple comparisons.

β-diversity and phylogenetic statistical analyses.

In order to determine the relative contributions and amount of variance explained by host predictor variables, permutational multivariate analyses of variance (PERMANOVA) tests based on Bray-Curtis, Jaccard, and Unifrac distance matrices were run in the R vegan package [93]. Bray-Curtis/Jaccard distances were estimated using vegan. Weighted and unweighted Unifrac distances were estimated using phyloseq. Bray-Curtis and weighted Unifrac distances take into account the abundances of bacterial taxa while Jaccard and unweighted Unifrac metrics only consider their presence or absence. Both UniFrac metrics utilize information on the phylogenetic diversity of bacterial members when calculating microbiota similarity. Microbiota similarity and groupings across samples were visualized via Principal Coordinates Analysis (PCoA) plots.

To test for phylosymbiosis, i.e. the congruence between host phylogenetic relatedness and gut microbiota similarity, mean divergence times (mya) were calculated between every pair of host species in R. First, we retrieved 1000 phylogenetic trees that included all species of Artiodactyla and with molecular data African elephants (*Loxodonta Africana*) from Upham's et al. (2019) Mammalian supertree [94]. The trees were randomly sampled from the posterior distribution of Upham's supertrees (Mammals birth-death tip-

dated DNA-only trees) using the VertLife online resource (http://vertlife.org/). Each tree was pruned to include only the species in this study, and branch lengths (i.e. divergence times between each pair of host species) were extracted using the ape package [95]. All 1000 trees showed the same phylogenetic relationships among the study species. Matrices of mean divergence times were estimated from the 1000 trees. To determine the strength of the phylosymbiosis signal, mantel tests from the vegan package were run on distance matrices built from the gut microbiota and host divergence time data, using 999 permutations and Spearman correlations. We visualized these results by plotting gut microbiota similarity (0-1) against host phylogenetic divergence time (mya) in ggplot2. We also constructed a consensus phylogeny of the host species.

Finally, to detect the bacterial taxa most indicative of given host families, bovid species or dietary guilds, we used the indicspecies package [96], which calculates an indicator value for each bacterial taxon based on its prevalence in a given group and absence in others. Significance was assessed by permutation tests using 999 random permutations. The indicator values of statistically significant bacterial families were plotted as barplots in ggplot2.

Comparisons of Masai Mara and Laikipia herbivores.
In order to compare the gut microbiotas of Masai Mara (1°22′19″S, 34° 56′17″E) herbivores to the gut microbiotas of their conspecifics in Laikipia (0°17′33″N, 36° 53′55″E), we concurrently processed the raw 16S rRNA gene sequences from the samples from this study and those from Kartizinel *et al.* [32] in DADA2. A total of eight herbivore species overlapped between the two studies: African buffalo, domestic cattle, Common eland, impala, giraffe, warthog, plains zebra, and African elephant. Samples available for download from Kartizinel *et al.* [32] and samples from our study were coincidentally both collected during wet seasons in their respective regions. For a list of all samples (N=305), and their associated meta data, see the *Availability of data and materials* section.

The bioinformatics processing and statistical analyses were performed as described above, with a few exceptions. In DADA2, forward and reverse reads were trimmed to 240bp and 150bp, respectively. Up to 2 errors were allowed per forward read and up to 4 errors per reverse read. To visualize gut microbiota compositions across species, heatmaps were constructed using pheatmap (v. 1.0.12) [97], which showed the relative abundances of the top 30 bacterial ASVs. To identify the strongest predictors of gut microbiota structure, we constructed three PERMANOVA models. The main model included host dietary guild, host species, host geographic region, sample month, and sample year as variables. A second model restricted analyses to within species (strata=species), and a third model was conducted within dietary guilds (strata=dietary guild). Testing for phylosymbiosis was conducted as described above.

DECLARATIONS

Ethical Approval and Consent to participate

Our research and procedures were most recently approved on April 16, 2019 (IACUC approval no. PROTO201900126) and comply with the ethical standards of Michigan State University and Kenya.

Consent for publication

Not applicable

Availability of data and materials

The 16S rRNA gene sequence data from this study were deposited in NCBI's Sequence Read Archive, under BioProject PRJNA656793 and accession numbers SAMN15803511-SAMN15803691. Sample metadata, data output by DADA2 (ASV table & ASV taxonomic classifications), and R scripts for analyses and figures included in this manuscript are available on Github (https://github.com/rojascon/Rojas_et_al_2020_African_herbivores_gut_microbiome).

Competing Interests

The authors declare that there are no competing interests.

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Author Contributions

K.R.T., K.E.H., and C.A.R. designed the study, C.A.R. and Mara Hyena Project field assistants collected the samples. C.A.R., S.R.B., and K.R.T. analyzed the data. C.A.R., K.R.T, and K.E.H. wrote the manuscript and all authors approved the final version of the manuscript.

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641 **Table 1. List of host study species and their associated metadata.**
642

Order	Family	Species	Dietary Guild	Fermenter type	Total Samples (N)	Analyzed samples (N)
Cetartiodactyla	*Bovidae*	African buffalo	grazer	foregut	18	17
Cetartiodactyla	*Bovidae*	Domestic cattle	grazer	foregut	14	13
Cetartiodactyla	*Bovidae*	Common eland	mixed feeder	foregut	8	8
Cetartiodactyla	*Bovidae*	Impala	mixed feeder	foregut	20	20
Cetartiodactyla	*Bovidae*	Kirk's dik dik	browser	foregut	37	28
Cetartiodactyla	*Bovidae*	Thomson's gazelle	mixed feeder	foregut	14	14
Cetartiodactyla	*Bovidae*	Topi	grazer	foregut	19	18
Cetartiodactyla	*Giraffidae*	Masai giraffe	browser	foregut	25	18
Cetartiodactyla	*Suidae*	Warthog	mixed feeder	hindgut	9	8
Perissodactyla	*Equidae*	Plains zebra	grazer	hindgut	5	5
Proboscidea	*Elephantidae*	African elephant	mixed feeder	hindgut	12	12

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645
646 **Table 2. Microbiota richness, evenness, and phylogenetic diversity varies with host family**
647 **and dietary guild.**

	Factor	Chao 1 Richness	Shannon diversity	Phylogenetic diversity
Across study species (N=161)	Host family	χ^2=52.86, **p<0.001**	χ^2=31.38 **p<0.001**	χ^2=18.65 **p<0.001**
	Host dietary guild	χ^2=79.13 **p<0.001**	χ^2=65.18 **p<0.001**	χ^2=50.67 **p<0.001**
	Mean rainfall over prior 2 weeks (mm)	χ^2=1.64 P=0.14	χ^2=0.88 p=0.34	χ^2=2.03 P=0.13
	Mean minimum T over prior 2 weeks (°C)	χ^2=2.01 p=0.15	χ^2=2.22 p=0.15	χ^2=4.20 **p=0.022**
	Mean maximum T over prior 2 weeks (°C)	χ^2=0.11 p>0.73	χ^2=0.54 p>0.73	χ^2=0.008 p>0.92
Within bovids (N=118)	Host species	χ^2=59.55 **p<0.001**	χ^2=11.39 **p=0.02**	χ^2=51.05 **p<0.001**
	Host dietary guild	χ^2=20.17 **p<0.0001**	χ^2=29.47 **p<0.0001**	χ^2=10.25 **p<0.01**
	Mean rainfall over prior 2 weeks (mm)	χ^2=2.94 p=0.08	χ^2=0.53 p=0.46	χ^2=1.96 p=0.16
	Mean minimum T over prior 2 weeks (°C)	χ^2=4.62 **p=0.03**	χ^2=1.77 p=0.18	χ^2=4.60 **p=0.03**
	Mean maximum T over prior 2 weeks (°C)	χ^2=0.71 p<0.39	χ^2=0.01 p<0.91	χ^2=0.78 p=0.37

648
649 Shown are the Chi-Sq. values and p-values for linear mixed effects models specifying host family, dietary
650 guild, rainfall, and temperature as a predictor variable, sample month as a random effect, and an alpha-
651 diversity metric as a dependent variable. Host fermentation type was included in the model, but had no
652 explanatory power. A similar model restricted to bovids was also constructed, and specified host species
653 instead of host family. Significant p-values (α=0.05) are bolded.
654

Table 3. Host family, species, and dietary guild structure the gut microbiota of African herbivores.

Analysis	Host factors	Bray-Curtis (% variance explained)	Jaccard (% variance explained)	Weighted Unifrac (% variance explained)	Unweighted Unifrac (% variance explained)
Across all host study species (N=165)	Host family	24.73, **p=0.001*** **	22.77, **p=0.001*** **	30.33, **p=0.001*** **	26.97, **p=0.001*** **
	Host species	10.42, **p=0.001*** **	9.5, **p=0.001*** **	5.3, **p=0.001*** **	8.14, **p=0.001*** **
	Host dietary guild	13.62, **p=0.001*** **	12.29, **p=0.001*** **	13.59, **p=0.001*** **	12.72, **p=0.001*** **
	rainfall (mm)	0.4, p>0.05	0.4, p>0.05	0.3, p>0.05	0.3, p>0.05
	min T (°C)	0.3, p>0.05	0.3, p>0.05	0.2, p>0.05	0.4, p>0.05
	max T (°C)	0.3, p>0.05	0.3, p>0.05	0.1, p>0.05	0.3, p>0.05
	sample month	1.6, **p<0.01** **	1.5, **p<0.01** **	1.5, p>0.05	1.5, **p<0.05***
Across bovids (N=122)	Host species	17.19, **p=0.001*** **	15.21, **p=0.001*** **	10.60, **p=0.001*** **	14.04, **p=0.001*** **
	Host dietary guild	21.20, **p=0.001*** **	18.54, **p=0.001*** **	21.79, **p=0.001*** **	19.42, **p=0.001*** **
	rainfall (mm)	0.5, p>0.05	0.5, p>0.05	0.5, p>0.05	0.5, p>0.05
	min T (°C)	0.7, p>0.05	0.7, p>0.05	0.5, p>0.05	0.9, p>0.05
	max T (°C)	0.4, p>0.05	0.5, p>0.05	0.4, p>0.05	0.5, p>0.05
	sample month	2.6, **p<0.01** **	2.5, **p<0.05***	1.79, p>0.05	2.6, **p<0.05***

Shown are the R^2 values (% variance explained) and p-values for PERMANOVA tests based on 4 types of distance matrices. Bray-Curtis and Weighted Unifrac distance matrices take into consideration the proportions of bacterial taxa, while Jaccard and unweighte Unifrac take into account their presence or absence. Both Unifrac distances account for phylogenetic relatedness among bacterial types. *p<0.05 **p<0.01 ***p=0.001. Significant p-values (α=0.05) are bolded.

Figures and legends

Figure 1. Gut microbiota composition of African herbivores. A) Stacked bar plots showing the relative frequency of 16S rRNA gene sequences assigned to each bacterial family (or order, if a family-level classification could not be assigned) across samples. Samples are grouped by host species, and each color represents a bacterial family. **B)** Bacterial families enriched in the guts of hosts from different families as determined by indicator species analysis. Differences in these taxa abundances can explain differences among the microbiota of different groups. **C)** Bacterial families enriched in the guts of hosts from different dietary guilds as determined by indicator species analysis.

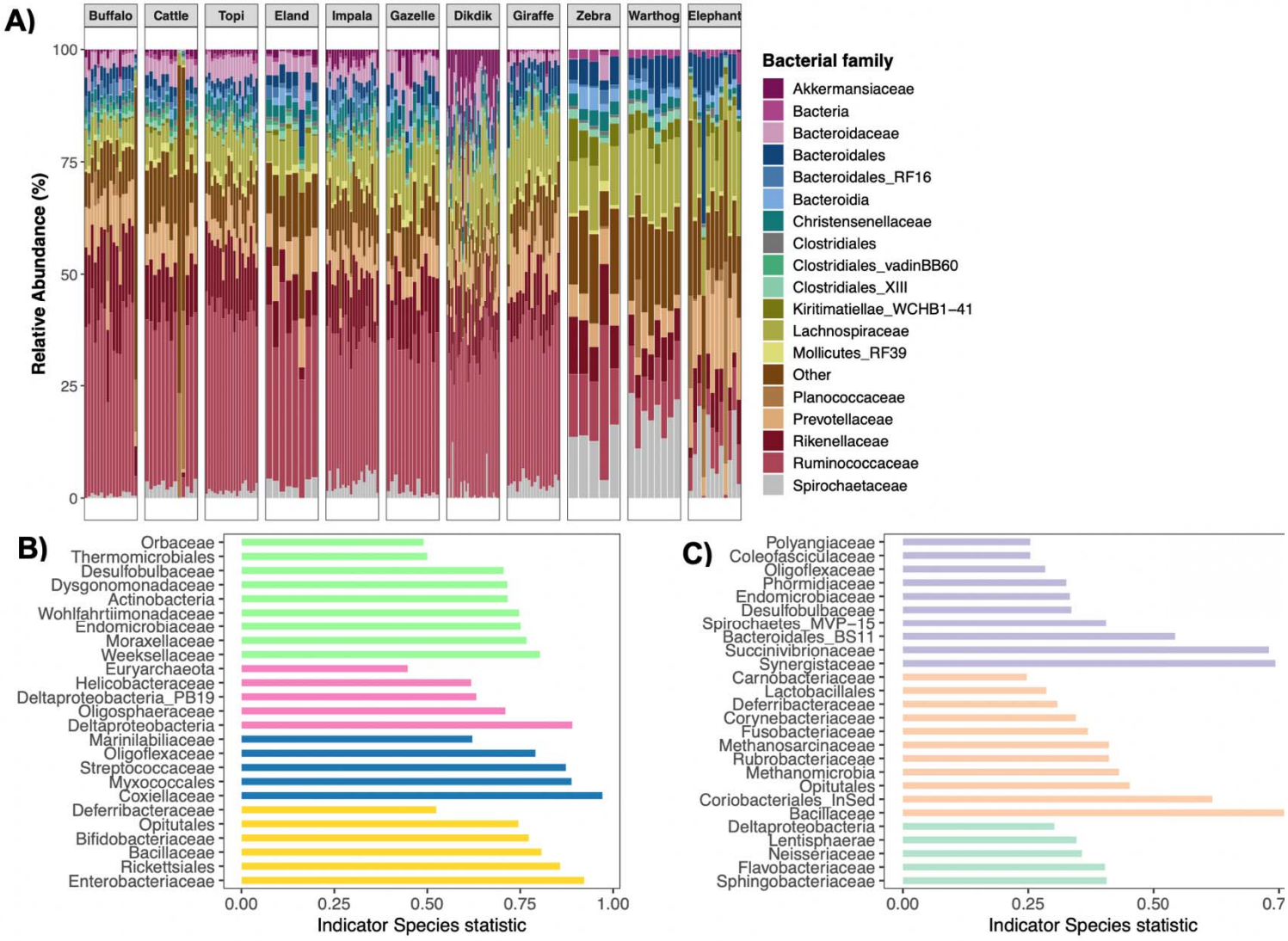


Figure 2. Host phylogenetic relatedness and dietary guild drive gut microbiota diversity and structure in African herbivores. A) Boxplots of microbiota evenness (Shannon diversity) among host families (left) and dietary guilds (right). Thicker dots represent outlier values. **B)** PCoA plots constructed from Bray-Curtis dissimilarity matrices. Each point represents a sample and is color-coded by host family (left) or host dietary guild (right). Closeness of points indicates high community similarity. The percentage of variance accounted for by each principal-coordinate axis is shown in the axis labels. **C)** Boxplots of microbiota evenness (Shannon diversity) among host species (left) and dietary guilds (right) within the family *Bovidae*. **D)** PCoA plots constructed from Bray-Curtis dissimilarity matrices of bovid species only. Each point is color-coded by host species (left) or host dietary guild (right).

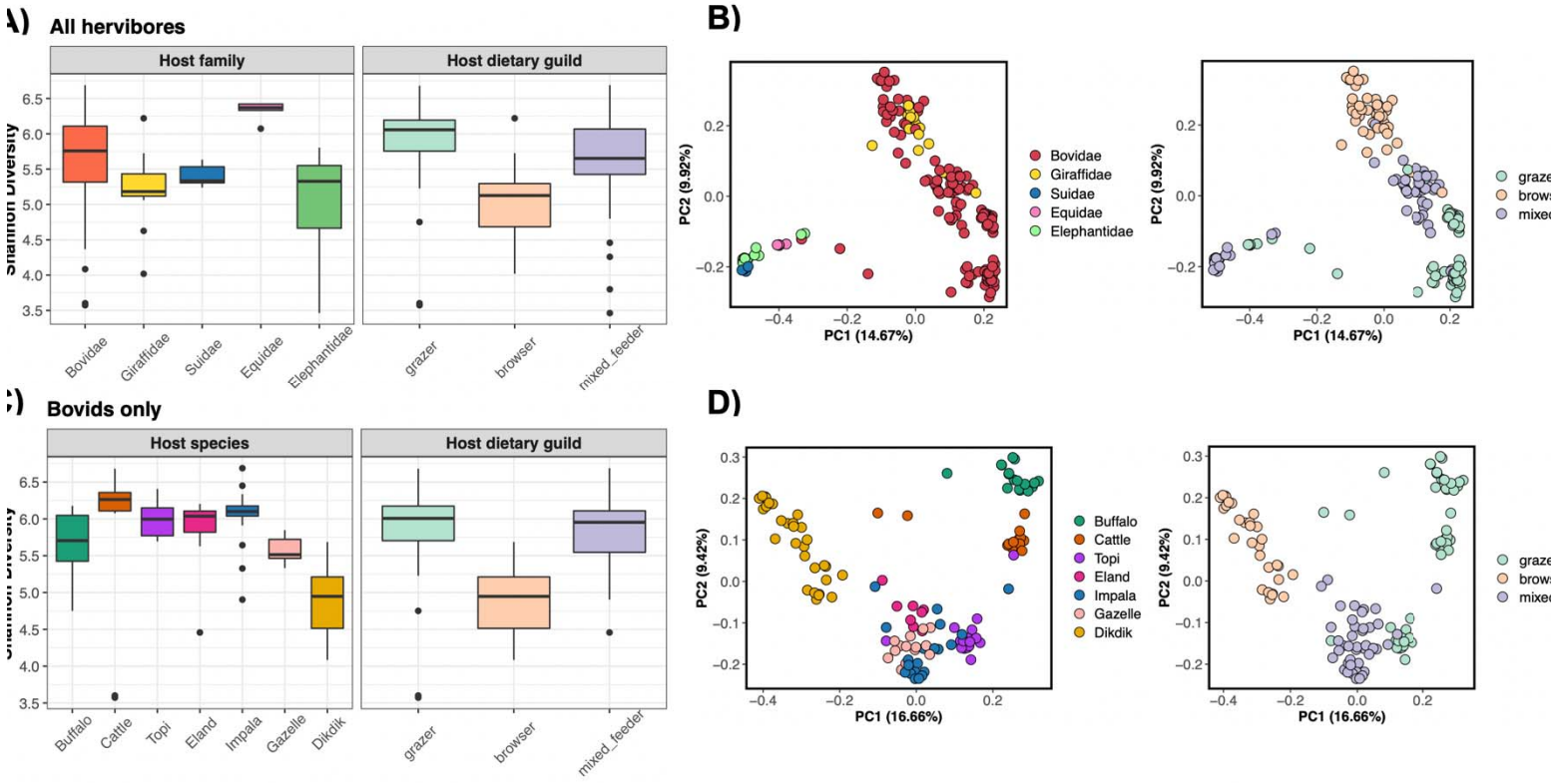
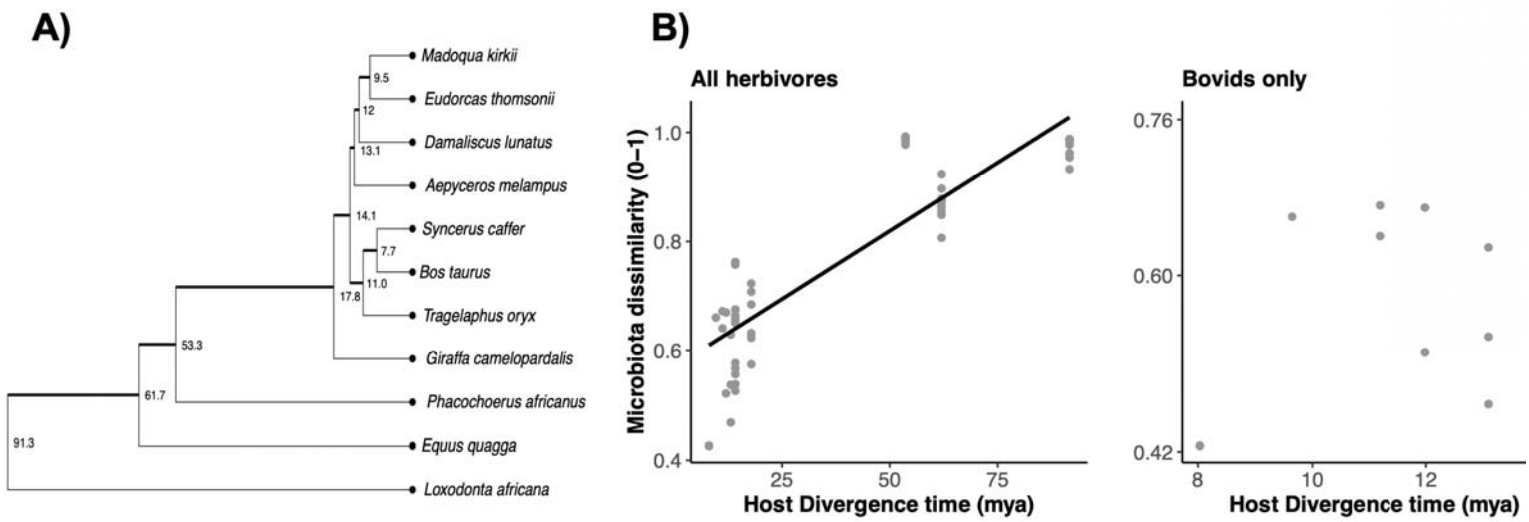
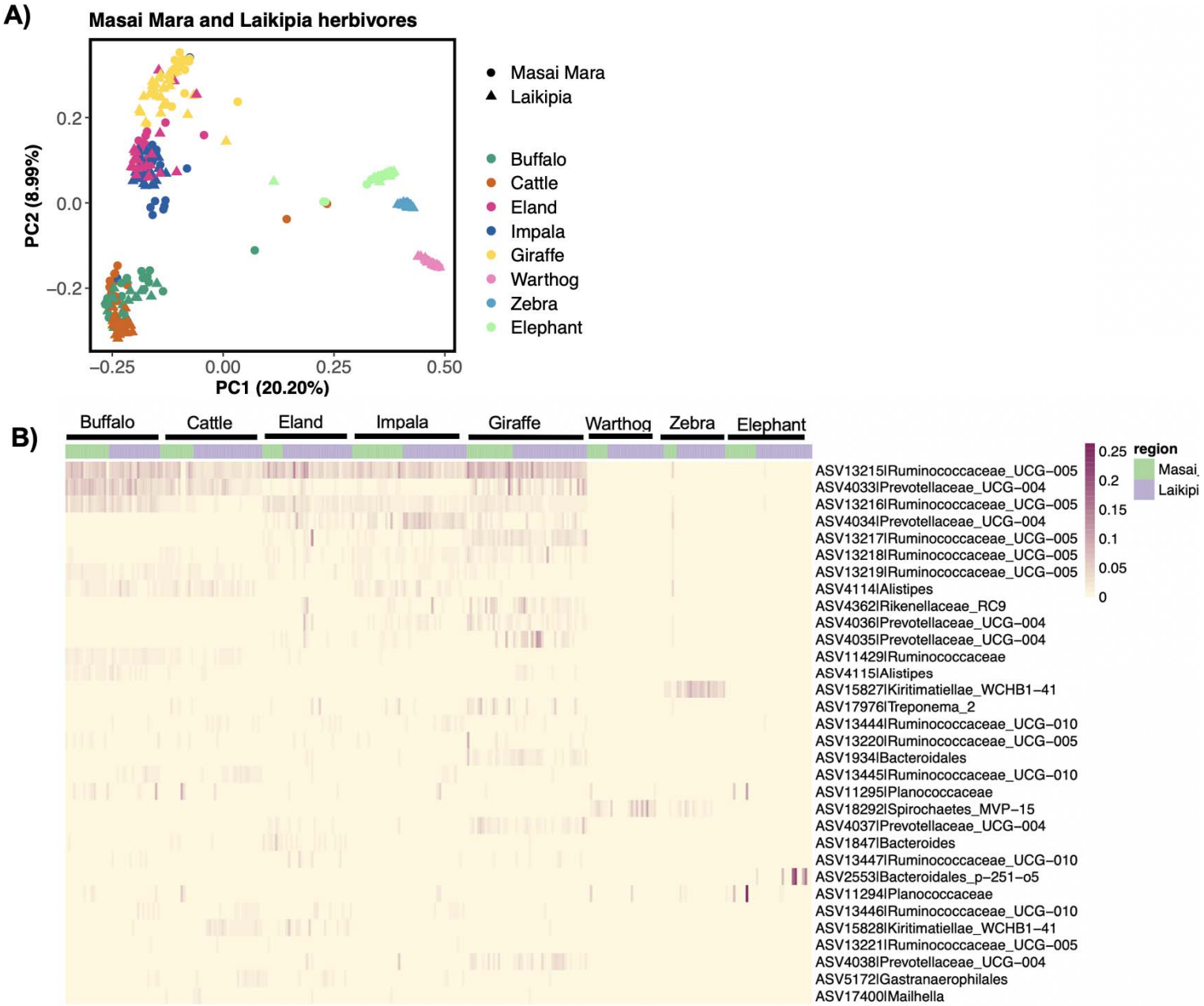


Figure 3. African herbivores exhibit patterns of phylosymbiosis. A) phylogenetic tree of host species obtained by pruning Upham's *et al.* 2019 Mammalian supertree. B) Plotted are pairwise host divergence times (in millions of years) vs. gut microbiota similarity (Bray-Curtis distances) across all sampled herbivores (left) and within the single host family *Bovidae* (right). A strong correlation is observed among the sampled herbivores (r=0.73, p<0.05), yet not within the family *Bovidae* (r=0.21, NS).



746 **Figure 4. Gut microbiotas of African herbivores are not shaped by host geography.** We
747 compared the gut microbiotas of eight species of herbivores residing in both the Masai Mara (this
748 study) and Laikipia (Kartzinel *et al.* 2019) regions in Kenya. **A)** PCoA plots constructed from Bray-
749 Curtis dissimilarity matrices. Each point represents a sample and is color-coded by host species.
750 The shapes represent the geographic region. Closeness of points indicates high community
751 similarity. The percentage of variance accounted for by each principal-coordinate axis is shown in
752 the axis labels. For PERMANOVA analyses, see Table S3. **B)** Top 32 most abundant bacterial ASVs
753 inhabiting the guts of Masai Mara and Laikipia herbivores. Heatmap showing the relative
754 abundances (proportions) of these top ASVs in eight species of herbivores. Samples are grouped by
755 host species and color-coded by host geographic region.



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Additional files

Additional file 1. Tables S1-S3. Table S1: Multiple-comparison testing of gut microbiota alpha-diversity among host families. **Table S2:** Multiple-comparison testing of gut microbiota alpha-diversity among host species in bovids. **Table S3:** PERMANOVA tests that included host species, geographic region, and dietary guild (Masai Mara and Laikipia herbivores). (.xlxs 17 KB).

Additional file 2. Figures S1-S4. Figure S1: Rarefaction curves of ASV richness. **Figure S2:** Stacked bar plots showing relative abundances of top bacterial phyla. **Figure S3:** Stacked bar plots showing relative abundances of top bacterial genera. **Figure S4:** PCoA ordinations showing sample clustering within each dietary guild (grazers, browsers, mixed-feeders). (.pdf 8.83 KB).

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