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

Running title: Gut microbiota of African 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 taxonomy accounted for more variation in the gut microbiota (30%) than did host dietary guild (10%) or sample month (8%). Overall, similarity among gut microbiotas increased with host phylogenetic relatedness ($r=0.73$), yet this relationship was not consistent among seven closely related Bovid host species ($r=0.21$ NS). Within bovinds, host dietary guild explained more of the variation in the gut microbiota than did host species. Lastly, while we found that the gut microbiotas of herbivores residing in southwest Kenya converge with those of distinct populations of conspecifics from central Kenya, fine-scale differences in the abundances of bacterial amplicon sequence variants (ASVs) between individuals from the two regions were also observed. Overall, our findings suggest that host phylogeny and taxonomy strongly structure the gut microbiota, but these gut microbial communities could be furthered modified by host ecology, especially among closely related host species.

Background

The gut microbiota performs critical roles for its host and is essential to host functioning. In mammals, resident gut microbes 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 host performance, research has focused on determining the forces that shape its assembly and composition. Decades of research show that across vertebrate hosts, the gut microbiota is predominantly shaped by host phylogeny and ecology. Closely related host species tend to have more similar gut microbiotas than more distantly related host species [8–12] and this congruence between host phylogenetic relatedness and gut microbiota similarity is termed “phylosymbiosis” [13–15]. However, gut microbiotas can also be further shaped by their host’s ecology, including their host’s diet, habitat, and geographic location [16–19]. Thus, although both of these host factors may shape the gut microbiota, their relative contributions might be influenced by a variety of variables including the taxonomic breadth of the host species surveyed, and the diversity of host habitats, diets, and physiologies represented.

Similarity in the gut microbiota among individuals could be due to hosts providing potential colonizing microbes with similar ecological niches [20, 21]. This could arise from overlap in diet, habitat, physiology, and behavior among hosts, as well as from shared evolutionary history. Thus, if phylosymbiosis is observed, both host phylogenetic relatedness and ecology could be contributing to the pattern. A diversity of studies have disentangled the effects of these two factors and have shown that phylosymbiosis can be observed among hosts that share habitats or diets, and 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 [22]. 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 [23]. Among hosts with overlapping diets, gut communities still exhibit patterns consistent with phyllosymbiosis, as has been documented for folivorous primates [9]. Furthermore, in 33 species of sympatric herbivores from the Laikipia region in central Kenya, host phylogenetic relatedness strongly predicted gut microbiota composition ($r = 0.91$) and was weakly correlated with host diet ($r = 0.28$) [24], suggesting that convergence of gut microbiotas among closely related hosts was not due to similarities in their diet.

Here, we build upon this work and use 16S rRNA gene sequencing to determine the relative influences of host phylogenetic relatedness and host ecology 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 (Table 1). These species represent 5 mammalian families (Bovidae, Elephantidae, Equidae, Giraffidae, and Suidae) and three dietary guilds: grazers, browsers, and mixed-feeders. Furthermore, we compare the gut microbiotas of conspecific herbivores from the Masai Mara and Laikipia to determine the extent to which host geography and/or local habitat influence gut microbiota composition and patterns of phyllosymbiosis. The two regions differ in their altitude, vegetation, mammal densities, and degree of human disturbance [25–29], any of which could potentially affect the gut microbiota compositions of their resident herbivores.

Specifically, we surveyed the gut microbiotas of 11 species of herbivores to 1) determine the relative contributions and amount of variance in the gut microbiota that is explained by host taxonomy (family and species) and host diet, 2) ascertain whether phyllosymbiosis is observed across broad host taxonomic scales (i.e. among all study species) and lesser host taxonomic scales (i.e. among 7 closely related Bovid species), and 3) examine the influences of host phylogenetic relatedness and host ecology (diet and geography) on the gut microbiota of conspecific Masai Mara (this study) and Laikipia herbivore hosts [24]. Collectively, our findings elucidate the factors shaping the gut microbiota of hosts at greater and lesser taxonomic scales.

Results

Aim 1: Determine the amount of variance in the gut microbiota explained by host taxonomy and host diet

Microbiota composition. Our analyses showed that some bacterial taxa were widely shared among host species and dietary guilds, whereas others were abundant only in particular host species. All herbivore gut microbiotas were dominated by two bacterial phyla, *Firmicutes* (51% average relative abundance across samples), and *Bacteroidetes* (32%) (Figure S1). The most abundant bacterial families were *Ruminococcaceae* (30.8%), *Rikenellaceae* (11.4%), *Lachnospiraceae* (10.9%), and *Prevotellaceae* (8%) (Figure 1A). Prevalent bacterial genera included *Alistipes*, *Bacteroides*, *Ruminococcus*, and *Treponema* (Figure S2). A total of 10 out of 11, 930 (0.08%) Amplicon Sequence Variants (ASVs) were present in 90% of samples pooled across all host species; 7 were assigned to the family *Ruminococcaceae*, 1 to *Peptococcaceae*, and 2 to *Lachnospiraceae* (*Agathobacter*). According to a blast search against the NCBI nucleotide database, sequences from the 7 *Ruminococcaceae* ASVs were highly similar to sequences from uncultured *Ruminococcaceae* strains, uncultured rumen bacteria, and uncultured anaerobic bacteria.

Of these 10 ASVs, only two were abundant across samples (e.g. ASV10543 *Ruminococcaceae*), five were modestly abundant in specific host species (e.g. ASV71 *Agathobacter* in elephants), and 3 were present at very low abundances in all samples (e.g. ASV7824 *Peptococcaceae*) (Figure S3). The latter 3 ASVs do not appear to be contamination introduced during DNA extraction and sequencing, as these sequences are highly similar to those found in rumen and fecal samples.

Nonetheless, variation in gut microbiota compositions among host species and dietary guilds was clearly evident. Indicator species analysis showed that the gut microbiotas of elephants were significantly associated with *Endomicrobiaceae* and *Desulfobulbaceae*, those of Zebras with *Helicobacteraceae* and *Deltaproteobacteria*, and those of warthogs with *Myxococcales* and *Coxiellaceae* (Figure 1B). Giraffe gut microbiotas were highly associated with *Enterobacteriaceae*, *Bifidobacteriaceae*, and *Bacillaceae*. Similarly, there were bacterial types that were indicative of specific dietary guilds. Grazer gut microbiotas were characterized by *Sphingobacteriaceae*, *Flavobacteriaceae*, *Neisseriaceae*, and *Lentisphaeria* (Figure 1C). Browser gut microbiotas had 11 indicator bacterial taxa, including *Bacillaceae*, *Coriobacteriales*, *Methanomicrobia* and *Rubrobacteriaceae*. Lastly, the gut microbiotas of mixed feeders were highly associated with *Synergistaceae*, *Succinivibrionaceae*, and *Bacteroidales*, among other bacteria.

More fine-scale analysis of the presence and absence of bacterial ASVs also revealed that the gut microbiotas of our studied herbivores contained microbes that were biased towards particular host species. These were bacterial ASVs that were present in 75% samples for that host species, and absent in 97% of other samples. Buffalo, cattle, topi, and impala gut microbiotas mostly contained ASVs that were present in other herbivores, as <3% of their ASVs were biased to any of these host species. Between 4% and 8% of ASVs comprising the gut microbiota of dik-diks, eland, elephant, Thompson's gazelle, and giraffe were biased towards these host species. Warthogs and zebras however, harbored more unusual microbiotas, as 70-77% of their ASVs were rarely detected in the guts of the other African mammals.

Microbiota alpha-diversity : Gut microbiota richness, evenness, and phylogenetic diversity also varied with host taxonomy (family and species) and dietary guild (Table 2, Figure 2). Post-hoc comparisons revealed that hosts from the Suidae and Elephantidae families generally harbored less rich and less even gut communities than the other surveyed host families (Figure 2A; Table S1). Moreover, equids harbored more phylogenetically diverse gut communities than all other herbivores (Table S1). Across the three alpha-diversity metrics, browsers had less diverse gut microbiotas than grazers or mixed-feeders (Figure 2A, Table S2). Within a single host family (i.e. Bovidae), the gut microbiotas of buffalo, dikdiks, and gazelles were less rich, even, and phylogenetically diverse than those of all other sampled bovids (Figure 2B, Table S3). In bovid hosts, browsers also had less diverse gut microbiotas than grazers or mixed-feeders (Figure 2B, Table S3).

Additionally, we determined whether *similarity* in gut microbiota alpha-diversity was correlated with a *quantitative measure* of host diet, while accounting for variation in the gut microbiota associated with host phylogenetic relatedness. Specifically, we used previously published dietary C4 (%) data [30–33] from these 11 herbivore species as our numerical measure of diet (Table S4). C4 (%) values quantify the degree of grazing by the host and

reflect the proportion of an animal's diet composed of C4 monocotyledon grasses relative to C3 trees, shrubs, and forbs. Similarity in gut microbiota *evenness* (Shannon diversity) increased with host overlap in dietary C4, but this was not true of gut microbiota richness or phylogenetic diversity (partial Mantel Chao1 $r=0.03$, $p=0.34$; Shannon $r=0.35$, $p=0.028$, PD $r=0.097$, $p=0.25$).

Microbiota beta-diversity: Across the surveyed herbivores, host family explained on average ~23% of the variation in gut microbiota structure, followed by host dietary guild (10%), sample month (8%), and host species (7.5%) (PERMANOVA analyses, 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. Therefore, for brevity, we only present PCoA ordination plots using the Bray-Curtis index. These plots show that gut microbiotas primarily partition by host family, and also secondarily by host dietary guild (Figure 3A).

Within Bovidae, host dietary guild was a slightly stronger predictor of the gut microbiota than host species or sample month. On average, host dietary guild accounted for 17.3% of the variation, whereas host species explained 12.2% of the variation, and sample month contributed to 7.3% of the variation (PERMANOVA analyses, Table 3). PCoA ordinations showed that the gut microbiotas of bovids clustered by host dietary guild and host species (Figure 3B). When controlling for host dietary guild and sample month, the microbiota is highly-species specific and host species accounts for 40% of the observed variation across samples (PERMANOVA: Bray-Curtis $R^2=0.42$; Jaccard $R^2=0.39$; Weighted Unifrac $R^2=0.40$, Unweighted Unifrac $R^2=0.41$; all $p=0.001$) (Figure S4).

Aim 2: Ascertain whether phylosymbiosis is observed among herbivore hosts at broad and lesser taxonomic scales

Although a dendrogram of gut microbiota similarity did not closely reflect host phylogeny (Figure 3C), gut microbiota similarity did increase overall with host phylogenetic relatedness among the 11 species of herbivores (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 $r=0.72$, $p=0.007$) (Figure 3D). The gut microbiotas 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). Despite warthogs being more closely related to bovids than to elephants and zebras, they had highly dissimilar communities from all of the herbivore species examined, and pairwise comparisons that include this host species deviate from the trendline (~1.0 Bray-Curtis dissimilarity, Figure 3D).

Importantly, at a lesser host taxonomic scale, among closely related host species in the Bovid family, we did not find a consistent relationship between gut microbiota similarity and host phylogenetic relatedness (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 $r=0.16$, $p=0.08$) (Figure 3D). However, when taking into account fine-scale variation associated with host diet (%C4) (Table S4), gut microbiota similarity is modestly correlated with host phylogenetic relatedness (partial Mantel Bray-Curtis $r=0.43$, $p=0.013$; Jaccard $r=0.35$, $p=0.016$; Unifrac $r=0.33$, $p=0.017$; Unweighted Unifrac $r=0.29$, $p=0.017$). When repeating this analysis on the dataset that includes all herbivore hosts, the original finding remained unchanged; accounting for fine-scale variation in host %C4 does not affect the phylosymbiosis pattern observed at a broad host taxonomic scale (partial Mantel Bray-Curtis $r=0.77$, $p=0.002$,

Jaccard $r=0.72$, $p=0.007$, Unifrac $r=0.75$, $p=0.004$, Unweighted Unifrac $r=0.72$, $p=0.01$). In summary, we observed phyllosymbiosis among hosts spanning a broad taxonomic scale involving multiple families, but not consistently among hosts spanning a lesser taxonomic scale involving only members of the Bovidae family.

Aim 3: Examine influences of host phylogenetic relatedness and host ecology on the gut microbiota of conspecific hosts

Lastly, we further analyzed the influences of host phylogenetic relatedness and host ecology (diet and geography) on the gut microbiota of 8 herbivore species from two distinct populations in the Masai Mara (this study) and the Laikipia region, respectively (Kartzinel *et al.* [24]). The eight herbivore species overlapping both studies were African buffalo, domestic cattle, common eland, impala, giraffe, plains zebra, common warthog, and African elephant.

We found that gut microbiota structure varied little among hosts from the two geographic regions, as this factor accounted for <3% of the variation in the gut microbiota (PERMANOVA analyses, Table S5). The gut microbiotas were primarily structured by host species and host dietary guild, which explained on average, 38% and 11% of the variation, respectively (PERMANOVA analyses, Table S5); sample month explained an additional 7% of the variation. Ordination plots confirm these findings and demonstrate that samples primarily cluster by host species (Figure 4A), although some separation of samples based on host geographic region is also observed, particularly among cattle, impala, and giraffe.

For this dataset, a total of 3 ASVs out of 18,039 (0.01%) were present across 90% of samples; two were classified as *Ruminococcaceae*, and 1 as *Lachnospiraceae*. All 3 ASVs were among the 10 ASVs present across 90% of Masai Mara samples. To further compare the gut microbiotas of conspecific hosts, we analyzed the relative abundances of the 32 most abundant ASVs in the dataset. A heatmap of these 32 ASVs demonstrate that there were ASVs that were found at comparable proportions in conspecific hosts, as well as ASVs that were differentially abundant among conspecific hosts (Figure 4B, Table S6). For example, ASV4033 *Prevotellaceae* is similarly abundant between Masai Mara and Laikipia hosts, but ASV15828 *Kiritimatiellae* appears to be enriched in cattle from Laikipia compared to cattle from the Masai Mara. Linear mixed models that statistically compared the relative abundances of these ASVs between the two groups showed that 13 of the 32 ASVs were actually differentially abundant between conspecifics (Table S6).

To extend these analyses and identify additional ASVs that may be differentially abundant among conspecifics, we also conducted Linear discriminant analysis Effect Size (LEfSe) for each host species. Roughly 30% of the ASVs in the gut microbiotas of each host species were enriched in hosts from one population relative to the other (Table S7). ASVs that were typically enriched were classified as *Ruminococcaceae*, *Lachnospiraceae*, *Rikenellaceae*, *Clostridiales*, *Bacteroidales*, and *Kiritimatiellae* (Figure 4C). Herbivores from Laikipia tended to be enriched in *Methanocorpusculum*, *Clostridiales*, and *Kiritimatiellae* ASVs relative to Masai Mara herbivores (Figure 4C). Masai Mara gut microbiotas were overrepresented by *Lachnospiraceae* and *Treponema* ASVs. Interestingly, hosts from both geographic regions could be enriched in taxonomically similar ASVs. For example, eland in Laikipia were enriched in 3 ASVs classified as *Prevotellaceae*, *Rikenellaceae*, and *Ruminococcaceae*, respectively, and eland from the Masai Mara were enriched in 3 different ASVs that were also classified as *Prevotellaceae*, *Rikenellaceae*, and

Ruminococcaceae (Figure 4C). These findings suggest that variation in the gut microbiotas of these herbivore conspecifics is observable at the level of specificity of bacterial ASVs.

Patterns consistent with phylosymbiosis were also observed in this combined dataset, despite herbivore hosts occupying different geographic regions in Kenya, and representing distinct populations. Gut microbiota similarity increased with host phylogenetic relatedness (mantel test Bray-Curtis $r=0.60$, $p=0.03$; Jaccard $r=0.64$, $p=0.04$; Weighted Unifrac $r=0.54$, $p=0.003$; Unweighted Unifrac $r=0.66$, $p=0.04$), although the strength of the correlation was less than that of Masai Mara herbivores only ($r=0.76$), or what was previously reported for Laikipia herbivores ($r=0.91$) [24]. Consistent with our earlier findings, phylosymbiosis was not evident among the four species of bovids that overlapped between the two studies (mantel test Bray-Curtis $r=0.30$, $p=0.25$; Jaccard $r=0$, $p=0.5$; Weighted Unifrac $r=-0.52$, $p=0.91$; Unweighted Unifrac $r=-0.49$, $p=0.83$). When taking into account gut microbiota variation attributable to host diet (% dietary C4), both findings remained unchanged (partial Mantel test all herbivores: Bray-Curtis $r=0.60$, $p=0.03$, Jaccard $r=0.64$, $p=0.04$, Weighted Unifrac $r=0.53$, $p=0.03$, Unweighted Unifrac $r=0.65$, $p=0.05$; bovids only: Bray-Curtis $r=0.36$, $p=0.16$, Jaccard $r=0$, $p=0.5$, Weighted Unifrac $r=-0.52$, $p=0.87$, Unweighted Unifrac $r=-0.61$, $p=0.83$).

Discussion

Principal findings of study. The primary purpose of this study was to determine the relative contributions of host phylogenetic relatedness and dietary guild in structuring the gut microbiotas of 11 species of sympatric African herbivores. We also compared the gut microbiotas of herbivores from the Masai Mara to herbivores from Laikipia, Kenya to determine the extent to which two distinct populations of identical herbivore species varied in their gut microbiotas. We found that gut microbiotas were highly species-specific, but also varied with host ecology, including host dietary guild and sample month, particularly in closely related Bovid species. Furthermore, gut microbiota similarity increased with host phylogenetic relatedness at broad host taxonomic scales, but at lesser taxonomic scales, the phylosymbiosis signal was only apparent after accounting for fine-scale variation attributable to host diet (%C4). Lastly, although the gut microbiotas of conspecific herbivore hosts converged and primarily clustered by host species, differences between conspecifics in the relative abundances of their bacterial ASVs were also observed. Collectively, our findings suggest that mammalian gut microbiotas are strongly shaped by host phylogenetic relatedness and taxonomy, but they can be further modified by host ecology, including host diet and geography.

Aim 1: Determine the amount of variance in the gut microbiota explained by host taxonomy and host diet

Across the surveyed herbivores, gut microbiota composition, diversity, and structured varied with host taxonomy. Species-specificity of the gut microbiota is widespread, and is commonly reported in the majority of comparative gut microbiome studies [11, 34–36]. Host species may vary in their body size, behavior, neuroendocrine system, immune function, and metabolism, any of which could potentially shape the structure of their gut microbiotas [37–40]. When comparing gut microbiota alpha-diversity, results showed that warthogs and elephants harbored less diverse gut communities than the other sampled herbivores. Due to their omnivory, warthogs have a greater dietary breadth than the other studied herbivores, yet they harbored less diverse microbiotas. This is in accordance with prior findings, which report that the most diverse diets do not always

correlate with the most diverse gut microbiotas [24, 41, 42]. Furthermore, analyses showed that browsers had the least diverse gut microbiotas, potentially because they consume vegetation that has a higher lignin content and a lower fiber digestibility than grass [43]. Specialized bacterial metabolisms may be required to digest this tougher plant material. Additionally, group size has been shown to correlate with gut microbiota diversity [44, 45], and the browsers in our study (giraffes, dikdiks) forage in smaller groups than grazers (buffalo, zebras) and mixed-feeders (gazelles, impala), which forage in herds. Frequent social interactions and interactions with a greater number of individuals is known to promote species richness in individual gut microbiotas [45, 46].

Similar to findings from a plethora of microbiome studies, the gut microbiota structure of the studied herbivores also varied with host diet. Hosts from each dietary guild consume food sources that vary in their structure, chemistry, and nutrition quality; which require morphological, physiological and behavioral adaptations [47, 48]. For example, grazers mostly feed on grasses, which have thicker cell walls, a lower protein content, and use the C₄ photosynthetic pathway compared to browse (e.g. leaves, shrubs, and woody vegetation), which have a higher protein content, and use C₃ photosynthesis [47, 48]. To efficiently extract energy from these different food sources, browsers and grazers evolved adaptations in their salivary chemistry, tooth morphology, gut structure, and speed of digestion [49, 50]. These adaptations, along with the actual nutrients hosts are providing to their microbes, potentially contribute to gut microbiota divergence among hosts from different dietary guilds.

Despite differences in the gut microbiota among host species and dietary guilds, there were some features of the gut microbiota that were shared across individuals from multiple species. 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 [8, 51], equids [52], elephants [53], and giraffes [54]. *Ruminococcaceae* and *Lachnospiraceae* have also been found in the guts of folivorous primates [3] and in domestic pigs [55, 56]. Members of these bacterial families are responsible for digesting the cellulose, hemicellulose, lignin, and protein found in vegetation, and fermenting these into short-chain fatty acids (SCFAs) [57]. SCFAs represent usable forms of energy for the hosts [58] and 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 [59].

Interestingly, 7 of the 10 ASVs that were present in 90% of Masai Mara herbivores were classified as *Ruminococcaceae* and were sequences highly similar to uncultured *Ruminococcaceae* strains extracted from bovine, goat, and sheep rumens [60], suggesting that these “core” microbes could be functionally important for the host.

Aim 2: Ascertain whether phyllosymbiosis is observed among herbivore hosts at broad and lesser taxonomic scales

Our results showed that phyllosymbiosis was observed across a broad host taxonomic scale, among 11 species of herbivores living sympatrically in the Masai Mara. Patterns of phyllosymbiosis have been documented extensively in many vertebrate groups, including primates, rodents, ruminants, carnivores, reptiles, and insects [9–12, 24, 61, 62]. Evidence

of phyllosymbiosis among host species living in *sympatry* specifically, has been previously documented in seven species of deer mice [63], six species of Malagasy mammals [64], twelve species of lemurs [65], and nine species of diurnal, non-human primates [66].

The mechanisms and processes that yield patterns of phyllosymbiosis 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 colonized by taxonomically similar microbes due to similarities in their morphology, anatomy, digestive physiologies, and immune system components [37, 38, 67]. Specifically, related hosts may possess similar antimicrobial peptides and toll-like receptors that serve to filter the same bacterial clades from the environment [68, 69]. Closely related hosts may further develop immune tolerance via adaptive immunity to the same symbiotic, commensal, and transient microbes [68, 69]. 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 phyllosymbiosis. Overall, accumulation of differences in traits as hosts diverged from one another could potentially provide enough niche differentiation in the gut to promote the divergence of their symbiotic bacterial communities.

At a lesser host taxonomic scale, within our sampled group of closely related Bovid species in the Masai Mara, variation in the gut microbiota was more strongly associated with host diet than host species, and a pattern consistent with phyllosymbiosis was only apparent if fine-scale variation in diet (%C4) was accounted for. This phyllosymbiosis signal was weaker ($r=0.35$) than that observed for all herbivores ($r=0.73$). Similarly, other studies report that among closely related hosts, host ecology more strongly predicts the structure of the gut microbiota than host relatedness. For example, in lemurs (*Eulemur* spp., *Propithecus* spp.), phyllosymbiosis 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 [65]. 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 [70]. Because the bovids surveyed here are closely related, their gut microbiotas are already very similar, and variation can result from fine-scale differences in diet (proportions of grass vs. shrubs vs. trees consumed) [43, 71–73]. Nonetheless, some of the variation in the gut microbiota of bovids is not attributable to host diet and can be explained by host phylogenetic relatedness. Thus, even among closely related host species, both ecological and evolutionary forces shape gut microbial communities.

Aim 3: Examine influences of host phylogenetic relatedness and host ecology on the gut microbiota of conspecific hosts

While gut microbiota structure was primarily associated with host species in the combined Masai Mara and Laikipia dataset, differences in gut microbiota composition between conspecific hosts from the two populations were also evident. Herbivores of the same species may possess similar evolutionary trajectories, physiologies, and behaviors, and thus may be providing microbes with similar niches for colonization, which is why at a broad level their gut microbiotas converge. However, the two geographic regions do vary in their climate, soil geochemistry, plant communities, and resident herbivore species [25–29, 74], and potentially in their bacterial species pools, which could lead to the fine-scale microbiota differences among conspecifics. This finding was supported by our data; over 30% of ASVs were differentially enriched between Masai Mara and Laikipia hosts. Laikipia herbivores for

example, tended to be enriched in ASVs classified as *Methanocorpusculum*, *Clostridiales*, and *Kiritimatiellae*, while Masai Mara herbivores had an overrepresentation of ASVs belonging to *Lachnospiraceae* and *Treponema*. Abundances of the methanogenic *Methanocorpusculum* are related to forage type and geographic location in cattle [75], and in the mammalian gut, *Lachnospiraceae* are associated with a high-fat diet [76]. Furthermore, in the bovine rumen, *Treponema* degrade hemicellulose and their growth increases in the presence of pectin [77, 78], a carbohydrate abundant in non-woody plants. This suggests that differences in the ASV abundances between conspecific hosts likely reflect fine-scale differences in their diets and habitats. Interestingly, both *Clostridiales* and *Lachnospiraceae* are major microbial taxa of the mammalian gut and comprise fermentative bacteria that synthesize SCFAs from the hydrolysis of starches and sugars [57]; thus, conspecific hosts can be enriched in taxonomically distinct microbes that perform similar functions.

Notably, 3 “core” bacterial ASVs were present in > 90% of samples from the 8 species of herbivores sampled from two allopatric populations. The 3 ASVs were highly similar to sequences from uncultured *Ruminococcaceae* strains and other anaerobic, rumen bacteria, illustrating that these ASVs a) may be easily acquired from the environment, and b) could be essential for host digestion.

Lastly, we found that phylosymbiosis was also evident among conspecific African herbivores living in allopatry, 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 [12]. It is important to note that differences between the gut microbiotas of Laikipia and Masai Mara conspecifics could also be potentially attributable to differences in sampling, DNA extraction, and sequencing protocols between the two studies [79–81]. Collectively, our findings show that mammalian gut microbiotas converge among closely related host species and among conspecifics, but can be differentiated with variation introduced by the host’s ecology.

Conclusions

Our study showed that among 11 species of African herbivores living in sympatry, gut microbiotas are highly species-specific and exhibit patterns congruent with phylosymbiosis. However, these gut microbiotas are also shaped by their host’s ecology, and we observed differences between hosts from different dietary guilds. Furthermore, among eight species of herbivores residing in two geographic regions in Kenya, gut microbiotas were similar among hosts of the same species, but also exhibited fine scale differences in the abundances of their bacterial ASVs. Overall, these findings suggest that related hosts are providing microbes with similar niches for colonization, but these microbial niches are further shaped by host diet, geography, and local environmental conditions. Future studies should examine whether phylosymbiosis is evident at the functional level in the gut metagenomes of African herbivores and in metazoan taxa in general. The gut microbiotas between distantly related host species could be taxonomically dissimilar, but functionally similar due to the widespread functional redundancy observed in microbial metabolisms. These studies will be necessary to further our understanding of the processes and mechanisms potentially underlying patterns of phylosymbiosis.

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 vegetation. It has two rainy seasons (March-May and November-December, with annual rainfall >1000mm) [82], and most of our sampling took place during the rainy months, particularly during the month of March (Figure S5). 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. Because our sampling occurred before July, samples from wildebeest and zebras were limited.

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.* [24]. 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 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* [83–86] to classify our study species into grazers, browsers, and mixed-feeders. To obtain more fine-scale data on host diet, we also compiled dietary C4 (%) data for these herbivores from previously published studies (Table S4) [30–33]. Percent C4 values reflect the proportion of monocotyledon grasses consumed relative to trees, shrubs, and forbs.

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 [87] and Kozich *et al.* 2013 [88].

Sequence processing and bioinformatics.

Sequences from Masai Mara herbivore gut microbiotas were processed in R (v.3.6.2) [89] using the Divisive Amplicon Denoising Algorithm (DADA2) pipeline (v.1.14.1) [90] 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) [91] 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% ($\pm 11\%$) 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 (giraffes and dik-diks) 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.

Microbiota composition analyses

Statistical analyses and data visualization were completed in R unless otherwise stated. To visualize microbiota composition, stacked barplots were constructed in ggplot2 (v.3.3.2) [92]. These plots showed the bacterial phyla, families, and genera with average relative abundances greater than 1% across samples. We also identified the ASVs that were present in >90% of samples across all host species, and the relative abundances of these ASVs were displayed as heatmaps using the R pheatmap package (v.1.0.12) [93]. Sequences from the 10 ASVs were blasted against the National Center for Biotechnology Information (NCBI) Nucleotide database [60] to find similar biological sequences from known bacterial taxa. Furthermore, we also identified ASVs that were biased towards particular host species; these were ASVs that were present in >75% of the samples for a particular host species (e.g. giraffes) and absent in 97% of samples from the other host species.

To detect the bacterial taxa strongly associated with particular host families or dietary guilds, we used the R indicpecies package (v.1.7.9) [94], which calculates an indicator value for each bacterial taxon based on its prevalence in a given group and absence in others. A table of bacterial family relative abundances was used as input, and significance was assessed via permutation tests using 999 permutations ($\alpha=0.05$). Bacterial families with indicator values >0.4 were plotted in ggplot2.

Microbiota α -diversity statistical analyses

Prior to alpha-diversity analyses, we controlled for the potential influences of sequencing depth by subsampling all samples to 17,000 sequences using the mothur (v.1.42.3) [95] 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 S6) 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 in other mammalian gut microbiota studies [8, 96, 97].

Microbiota alpha-diversity was estimated using Chao1 Richness, Shannon diversity, and Faith's phylogenetic diversity in R. Chao1 Richness and Shannon indices were calculated using the phyloseq package (v.1.33.0) [98]. To obtain measures of Faith's Phylogenetic Diversity (PD), we constructed a phylogenetic tree of ASV sequences using phangorn (v. 2.5.5) [99] and calculated PD using the picante package (v.1.8.1) [100]. The effects of predictor variables on each measure of alpha-diversity across all samples were evaluated via linear mixed models (LMMs) using the lme4 package (v.1.1.23) [101], specifying host dietary guild and host family as fixed variables and sample month as a random effect. A similar model was also built for bovid samples, which included host species as a predictor in lieu of host family. The significance of each predictor variable was

determined by calculating likelihood ratio Chi-2 test statistics ($\alpha=0.05$) on the full models using the car package (v.3.0.7) [102]. These tests were followed by TukeyHSD post-hoc tests with Benjamini-Hochberg adjustments to control for multiple comparisons. Boxplots of microbiota alpha-diversity were generated in ggplot2.

To further quantify the influence of host diet on the three metrics of gut microbiota alpha-diversity, we conducted partial Mantel tests in the R vegan package (v.2.5.7) [103]. Specifically, we evaluated whether similarity in gut microbiota alpha-diversity was associated with similarity in dietary C4(%) after taking into account variation due to host phylogenetic relatedness. The 3 matrices used as input were i) a dissimilarity matrix of gut microbiota alpha-diversity, ii) a dissimilarity matrix of host %C4, and iii) a matrix of host divergence times.

Microbiota β -diversity analyses and testing for phylosymbiosis

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 using vegan. Bray-Curtis/Jaccard distances were estimated using vegan, whereas 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. PERMANOVA model #1 included sample month, host dietary guild, host family, and host species as predictors (in this order). Model 2 was identical, except it was restricted to the Bovid dataset and excluded the host family term. Model 3 quantified the effect of the host species predictor while controlling for dietary guild (strata=dietary guild). 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 African elephants (*Loxodonta Africana*) from Upham's et al. (2019) Mammalian supertree [104]. 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 R ape package (v.5.4.1) [105]. All 1000 trees showed the same phylogenetic relationships among the study species and matrices of mean divergence times were estimated from those trees. To determine the strength of the phylosymbiosis signal, we conducted mantel tests on gut microbiota dissimilarity matrices and matrices of host divergence times, setting 999 permutations and Spearman correlations. We also ran partial Mantel tests to evaluate the strength of the phylosymbiosis signal while taking into account gut microbiota variation attributable to diet. The 3 matrices used as input were i) a gut microbiota dissimilarity matrix, ii) a matrix of host divergence times, and iii) a dissimilarity matrix of host %C4 (Table S4).

We visualized the phylosymbiosis findings by plotting gut microbiota similarity (0-1) against host phylogenetic divergence time (mya) in ggplot2. We also constructed a

consensus phylogeny of our host species and compared it against a dendrogram of gut microbiota dissimilarity, which was calculated using hierarchical clustering with the R stats package [89] and plotted using the ape package.

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) (~ 290 km from the Masai Mara), we downloaded all publicly available sequences from Kartzinel *et al.* [24], and combined them with the raw 16S rRNA gene sequences from this study (Masai Mara herbivores). These were then processed together 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. The majority of samples from Kartzinel *et al.* [24] and from our study were collected during the wet seasons in their respective regions (Table S8), although, in general, Laikipia is more arid than the Masai Mara, with only 300-600mm precipitation annually [28, 106, 107]. 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, to better account for sequence quality. Up to 2 errors were allowed per forward read and up to 4 errors per reverse read. To identify the strongest predictors of gut microbiota structure, we constructed a PERMANOVA model that included sample month, host geographic region, host dietary guild, and host species as variables (in this order). PCoA ordinations and testing for phylosymbiosis were conducted as described earlier. To visualize gut microbiota compositions between Masai Mara and Laikipia herbivores, a heatmap of the 32 most abundant bacterial ASVs was constructed using R pheatmap. To statistically evaluate whether conspecific hosts differed in their abundances of these 32 ASVs, we ran 32 distinct LMMs in R, each specifying an ASV relative abundance as the dependent variable, host region as a fixed effect and host species as a random effect (to restrict analyses to within a host species). p-values were adjusted for multiple comparisons using the Bonferroni-Hochberg method. To extend this analysis to include more ASVs, we conducted Linear discriminant analysis Effect Size (LEfSe) [108] in the Galaxy platform [109] using default parameters. Only ASVs >0.01% average relative abundance across samples were included in the dataframe uploaded to Galaxy. ASVs that were enriched in hosts from one geographic region relative to the other were visualized via diverging dot plots in R with the ggplot2 package.

List of abbreviations used in this manuscript

Amplicon Sequence Variant (ASV)
 Linear Mixed Model (LMM)
 Permutational Multivariate Analysis of Variance (PERMANOVA)
 Principal Coordinates Analysis (PCoA)
 Linear discriminant analysis Effect Size (LEfSe)
 National Center for Biotechnology Information (NCBI)
 Short Chain Fatty Acids (SCFAs)
 University of Chicago at Illinois (UIC)
 Divisive Amplicon Denoising Algorithm (DADA2)
 Faith's Phylogenetic Diversity (PD)
 Millions of years (mya)

Institutional Animal Care and Use Committee (IACUC)

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), data obtained from LEfSe, and R scripts for all analyses and figures included in this manuscript are available on Github (https://github.com/rojascon/Rojas_et_al_2020_African_herbivores_gut_microbiome).

Supplementary Materials

Supplementary tables and figures can be found online at bioRxiv, alongside this manuscript submission.

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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Author's information

Not applicable

Tables

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

Order	Family	Species	Dietary Guild	Total Samples (N)	Analyzed samples (N)
<i>Cetartiodactyla</i>	<i>Bovidae</i>	African buffalo	grazer	18	17
<i>Cetartiodactyla</i>	<i>Bovidae</i>	Domestic cattle	grazer	14	13
<i>Cetartiodactyla</i>	<i>Bovidae</i>	Common eland	mixed feeder	8	8
<i>Cetartiodactyla</i>	<i>Bovidae</i>	Impala	mixed feeder	20	20
<i>Cetartiodactyla</i>	<i>Bovidae</i>	Kirk's dik dik	browser	37	31
<i>Cetartiodactyla</i>	<i>Bovidae</i>	Thomson's gazelle	mixed feeder	14	14
<i>Cetartiodactyla</i>	<i>Bovidae</i>	Topi	grazer	19	19
<i>Cetartiodactyla</i>	<i>Giraffidae</i>	Masai giraffe	browser	25	18
<i>Cetartiodactyla</i>	<i>Suidae</i>	Warthog	mixed feeder	9	8
<i>Perissodactyla</i>	<i>Equidae</i>	Plains zebra	grazer	5	5
<i>Proboscidea</i>	<i>Elephantidae</i>	African elephant	mixed feeder	12	12

Table 2. Microbiota richness, evenness, and phylogenetic diversity vary with host taxonomy and dietary guild.

	Factor	Chao 1 Richness	Shannon diversity	Phylogenetic diversity
Across study species (N=161)	Host family	$\chi^2=53.58$, p<0.001	$\chi^2=33.45$ p<0.001	$\chi^2=18.31$ p=0.001
	Host dietary guild	$\chi^2=79.027$ p<0.001	$\chi^2=73.72$ p<0.001	$\chi^2=52.61$ P<0.001
Within bovids (N=118)	Host species	$\chi^2=57.03$ p<0.001	$\chi^2=10.81$ p=0.02	$\chi^2=50.53$ p<0.001
	Host dietary guild	$\chi^2=19.55$ p<0.0001	$\chi^2=33.91$ p<0.0001	$\chi^2=10.67$ p<0.01

Shown are the likelihood ratio χ^2 test statistics obtained for linear mixed effects models specifying host dietary guild and host family as predictor variables, sample date as a random effect, and an alpha-diversity metric as a dependent variable. A similar model restricted to bovids was also constructed; it specified host species instead of host family. Significant p-values ($\alpha=0.05$) are bolded.

Table 3. Host taxonomy and dietary guild shape the gut microbiotas 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	22.33, p=0.001	20.59, p=0.001	26.38, p=0.001	24.23, p=0.001
	Host dietary guild	11.25, p=0.001	10.21, p=0.001	9.71, p=0.001	10.30, p=0.001
	Host species	9.40, p=0.001	8.6, p=0.001	4.45, p=0.001	7.23, p=0.001
	sample month	7.42, p=0.001	6.80, p=0.001	10.21, p=0.001	7.66, p=0.001
Across bovids (N=122)	Host dietary guild	18.26, p=0.001	15.91, p=0.001	18.35, p=0.001	16.77, p=0.001
	Host species	15.16, p=0.001	13.38, p=0.001	8.23, p=0.001	12.16, p=0.001
	sample month	7.56, p=0.001	7.03, p=0.001	7.59, p=0.001	7.17, p=0.001

Shown are the R^2 values (% variance explained) and p-values for PERMANOVA tests ($y \sim$ sample month + host dietary guild + host taxonomy) 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 unweighted Unifrac take into account only their presence or absence. Both Unifrac distances account for phylogenetic relatedness among bacterial types. Significant p-values ($\alpha=0.05$) are bolded.

Figure 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 significantly associated with particular herbivore families as determined by indicator species analysis. Differences in these taxa abundances can explain differences in the microbiota among the different groups. **C)** Bacterial families significantly associated with herbivores from different dietary guilds as determined by indicator species analysis.

Figure 2. Host taxonomy and dietary guild are associated with gut microbiota diversity in African herbivores. A) Boxplots of microbiota evenness (Shannon diversity) among host families and dietary guilds across all studied herbivores, and **B)** among host species and dietary guilds within the family *Bovidae*. Boxes that do not share any letters represent statistically significant comparisons; see Tables S1-S3 for all post-hoc comparisons. Thicker dots represent outlier values.

A) All herbivores

B) Bovids only

Figure 3. African herbivore gut microbiotas exhibit patterns consistent with phyllosymbiosis. A) 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. **B)** 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). **C)** Phylogenetic tree of host species (left) obtained from pruning Upham's *et al.* 2019 Mammalian supertree, compared against a dendrogram (right) of gut microbiota similarity using hierarchical clustering. **D)** Scatterplot of 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).

Additional files

Additional file 1. Tables S1-S8. Table S1-S3: Multiple-comparison testing of gut microbiota alpha-diversity among host families, species, and dietary guilds. **Table S4:** Previously published dietary %C4 data for the surveyed herbivores. **Table S5:** PERMANOVA tests that included sample month, geographic region, host dietary guild and host species for the combined Masai Mara and Laikipia dataset. **Table S6:** Linear models testing whether conspecific hosts differ in the relative abundances of 32 dominant ASVs. **Table S7:** Proportion of ASVs that were differentially enriched in herbivores from one geographic region compared to the other according to LEfSe. **Table S8:** Sample sizes for each month for the combined Masai Mara and Laikipia dataset (.xlsx 29 KB).

Additional file 2. Figures S1-S6. Figure S1-S2: Stacked bar plots showing relative abundances of top bacterial phyla and genera. **Figure S3:** Heatmap of the relative abundances of 10 widely-shared bacterial ASVs. **Figure S4:** PCoA ordinations showing sample clustering within a dietary guild (grazers, browsers, mixed-feeders). **Figure S5:** Stacked barplot of the proportion of samples from each host species that were collected each month (for the Masai Mara dataset). **Figure S6:** Rarefaction curves of ASV richness for the study samples (.pdf 1.9 MB).

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