

Captivity, diet and geography may impact the composition of animal gut microbiota

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SUMMARY The mammalian gut microbiota is influenced by various factors, such as geography, captivity and diet. Geography has been observed to cause variations within a single species, while captivity has been shown to disrupt gut microbial diversity. A major driver of the variation observed in geography and captivity has been accredited to changes in diet composition. Based on these observations, we hypothesized that all three factors will play a major role in shaping the gut microbial communities of captive and wild mammals. In this study, we used alpha and beta diversity metrics to observe the effects of geography and captivity in mammals, which led to studying diet in primates. We identified bacterial indicator taxa with regards to geography and captivity as well as conducted correlation analysis for diet. We observed patterns between geography and gut microbial composition, and hence, similar to previous studies, we conclude that geography influences the gut microbiome. Also in line with past findings, captivity was seen to change the microbial composition of animals such that convergence towards the human gut microbiome was observed. We were also able to demonstrate some correlation between food source and members of microbiota of various primates with findings in agreement with past studies of human and other primate gut microbiome.

INTRODUCTION

The gut microbiome is known to be imperative to normal development, digestion and reproduction in humans and animals (1). Previous studies have attributed various factors that could contribute to gut microbiota composition, such as geography, captivity and diet (2–4). Animals that live in close proximity of one another have been observed to have similar microbial compositions compared to animals that live further away (5, 6), and this pattern has been attributed to animals being exposed to the same environment, as well as similar availability of resources. As in both captive and wild conditions, the diet composition largely influences the composition of gut microbiota. In the wild, animals are influenced by multiple factors that determine diet composition, including seasonal food availability and climate shifts; which can then be further tied to the temperature, humidity and other geographical-dependent variables of a given area (5, 6). Geography and food availability are closely intertwined, as seasonal variations and aforementioned factors determine the type of plant and animal communities that grow within that region (7). Captivity is another factor that affects the gut microbiome; captive animals are subject to controlled environments vastly different from their wild counterparts, which has been shown to cause significant changes to their gut microbiome (8). The severity of captivity has also been shown to be correlated with gut microbiome disruption and humanization in captive primates (9).

Primate is a mammalian order of common omnivores including humans. In the wild environment primates consume a large variety of plant and animal-based food including: fruits, plants, seeds, invertebrates and sometimes small vertebrates (10). Depending on the combination, the diet can shift between that with a high amount of sugar from fruits, to high amounts of fiber from plants or high amounts of lipids from seeds. In their natural habitat, due to the shift in the availability of food sources mentioned above, primates are constantly pressured to adapt to a shifting diet composition which is also found to result in the changes in gut microbiota (11). In captivity, the differences in veterinary practices including medicine and diet supplementation are common factors impacting primate gut microbiota (10).

Such phenomena of captive animals demonstrating drastic shifts and developing humanized microbiota have been extensively studied in primates, but not in other mammalian

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species. Furthermore, most of the past microbiota studies in primates have been limited to a small number of species, rather than across multiple species or higher taxa (13, 14). Therefore, we were interested to see how geography, captivity and diet affect the gut microbiota of mammals. Based on previous studies, we developed a hypothesis for each factor. We hypothesize that: 1) Geography should play a role in shaping the gut microbiota, considering its importance in forming the local ecological environment including microbial, animal and plant communities 2) Captivity will cause humanization of the animal gut microbiome 3) The microbiota of various primate species will cluster based on similar diet composition and such effect may be a factor in the decrease in microbial diversity in captivity.

Overall, we aim to investigate the role of various factors that influence the composition of mammalian microbiota, specifically that of geography as it impacts the food availability. We also attempt to investigate the role of captivity in causing humanization of gut microbiome in mammals, and then to determine the influence of diet composition on primate gut microbiota. We observed clustering of animals at the same geographic location, as well as relation between geography and diet. With regards to captivity, convergence was shown to be not a universal phenomenon and driven by not a single dominant bacterial taxon, but multiple subdominant taxa. Primates exhibited clustering based on diet composition, and gut composition seemed to correlate with food sources ingested with specific bacterial classes correlating with high plant or high fruit diet.

METHODS AND MATERIALS

Sample processing. Animal fecal sample data consisting of V4 region of 16S rRNA amplicon sequences from the study “The Effect of Captivity on Mammalian Gut Microbiota” by McKenzie *et al.* was used for our study (8). Demultiplexed sequence data was imported into QIIME2, and prior to quality control and trimming with DADA2 (15), samples were subsetted according to each aim. (For investigating diet composition in primates, samples were subsetted for primates, and diet categories were created based on the original data set using the tidyverse package on R (16). For investigating geography, samples were subsetted into captive and wild animal categories and then by family or genus using QIIME2 (17). Sequences were trimmed to 140nt and low abundance sequences/rare ASVs along with mitochondrial and chloroplast sequences were removed. For the humanization study, human fecal data (V4 16S region) was downloaded from Qiita (“Human gut microbiome differentiation viewed across cultures, ages and families illumina”) and imported to QIIME2 for processing and analysis. The human data was preprocessed with Deblur and trimmed to 100nt. As such, animal data was processed and trimmed in the same manner. Animal and human data were subsetted and filtered separately, merged and were analyzed on QIIME2 as well as exported to R for further analysis.

Alpha diversity analysis. Alpha diversity measures were calculated using the Shannon diversity index on QIIME2. Unique sampling depths were chosen for each aim as the number and type of samples used.

Beta diversity analysis. Beta diversity measures were calculated using QIIME2. Bray-Curtis dissimilarity distance metric was used for the geography study, Unweighted UniFrac for captivity and Weighted UniFrac for diet. Principal-coordinate analyses were performed on QIIME2 and R, and visualized using Emperor and ggplot, respectively (18, 19). Testing beta diversity between categorical data was performed using PERMANOVA on QIIME2.

Taxonomic analysis. Taxonomic compositions were determined using the QIIME 2 naive Bayes classifier trained on the Greengenes 13_8 99% OTUs (for the captivity study, the classifier was trained on Greengenes 97% OTU). Data from QIIME2 was imported into R using phyloseq, and filtered for further analysis (20). Differential abundance was assessed with DESeq2 with an abundance cutoff of 0.1% (21). Statistical significance was determined using an adjusted p-value of 0.05. Indicator taxa was analyzed using multi-pattern level analysis with the R package indicspecies (22). The indicator statistic is a representation of the specificity and fidelity of the taxa identified to the location/species. A high score indicates high specificity and fidelity.

Correlation analysis. Scatter plots of relative abundance of bacterial classes against the proportion of plant or fruit in the diet were plotted in R using the ggplot package and its correlating functions. Pearson correlation statistics are computed in R studio using cor.test function.

RESULTS

Geography potentially influences gut microbiota. For our first hypothesis, we wanted to observe if taxonomically related animals living within the same location would group together. Using Bray-Curtis dissimilarity distance metric, we compared 1 family, 1 genus and 3 species, of which there were multiple sample collection sites. Ultimately, all animals analyzed were from the captive subset, as in the wild subset there were only samples that contained a single species at each location, so they were excluded to prevent confounding variables. Principal-coordinate analysis (PCoA) showed that there is a pattern of clustering based on location at the family and genus level, in the family *Rhinocerotidae* and genus *Equus*, respectively (Fig. S1A, B). This is also shown at the species-level with giant anteaters (*Myrmecophaga tridactyla*) and armadillos (*Oryzomys azer*) (Fig. 1). However, with gorillas (*Gorilla gorilla*), no distinct clustering was observed, notably in the Beauval Zoopark samples (Fig. S1C).

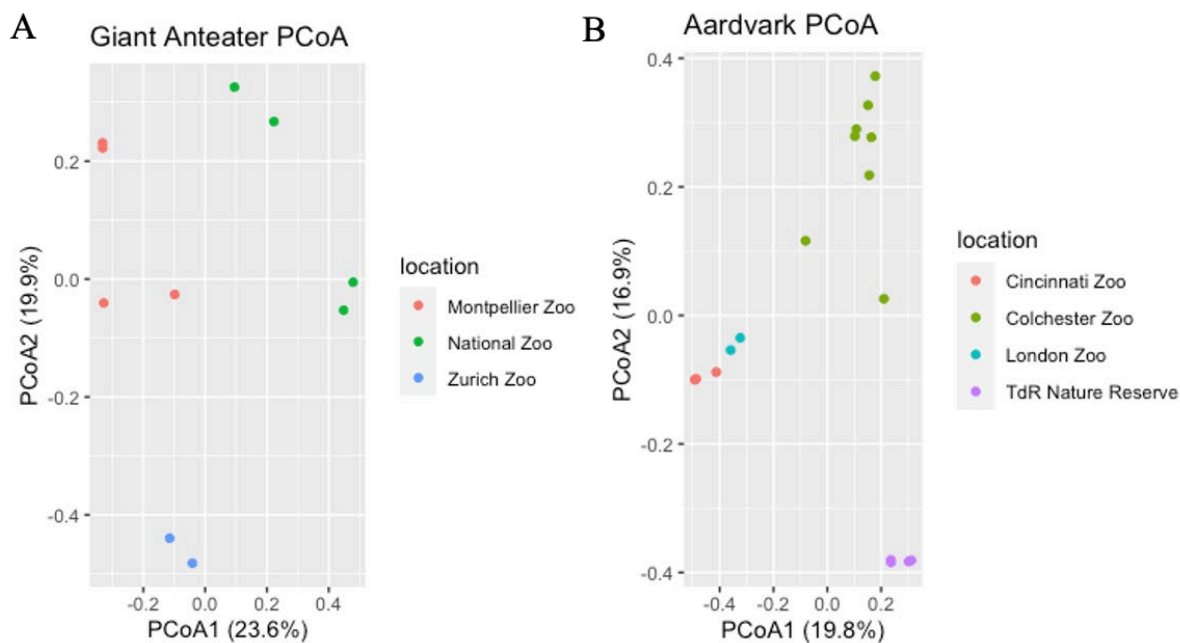


FIG. 1 Species-level principal coordinate analysis displays similar microbiota in myrmecophagous animals at different locations. PCoA plots of Bray-Curtis dissimilarity index showing gut microbial diversity across various locations in (A) giant anteaters and (B) armadillos. Pairwise PERMANOVA of Cincinnati Zoo to Colchester Zoo, and Colchester Zoo to TdR Nature Reserve showed statistical significance (q value < 0.05). No statistical significance was observed between other locations due to low sample size.

To examine differences in gut microbial composition, we looked at bacterial taxa present in specific sampling locations within a single animal species. Giant anteaters and armadillos had 3 and 4 sampling locations, respectively. Using multi-level pattern analysis, we examined indicator taxa at the family level. At Zurich Zoo, a majority of the identified taxa in giant anteaters belonged to the phylum Firmicutes, whereas Firmicutes was not detected in other sampling sites (Table S1). In armadillos, the phyla of indicator taxa were similar across locations. All locations contained members from the phyla Firmicutes and Bacteroidetes, and 3 out of 4 locations contained Proteobacteria (Table S2). Overall, bacterial taxa that appeared to be present at specific locations. Therefore, we conclude that geography influences the gut microbiome.

Captivity leads to convergence in some mammals but not others. We determined geography to be a driver of microbiome variation, and we sought to find out if captivity was another major contributor. More specifically, we wanted to find out if captivity leads to humanization of the animal gut microbiome. This phenomenon has been observed in primate species (9); as such, we asked whether a similar pattern could be observed in other mammalian species. To address this question, we compared the gut microbiome of captive and wild animals from 6 species across 4 Orders (namely, aardvarks, giant anteaters, lemurs, western gorillas, rhinoceros and zebras), to those of non-Western (Malawi and Venezuela) and Western humans (USA) using Unweighted UniFrac distance metric. We observed, upon examining the principal coordinates analysis plot, that despite the animals having distinct microbiomes, captivity causes them to converge towards the human microbiome, especially to that of non-Western humans (Fig. 2, S2). However, this did not seem to be a general phenomenon, as convergence was observed in most, but not all, of the animals. Although analysis including a broader range of species and larger sample size is needed in order to make any robust conclusion, our results suggest that convergence towards the human gut microbiome is not a generalizable pattern among captive animals.

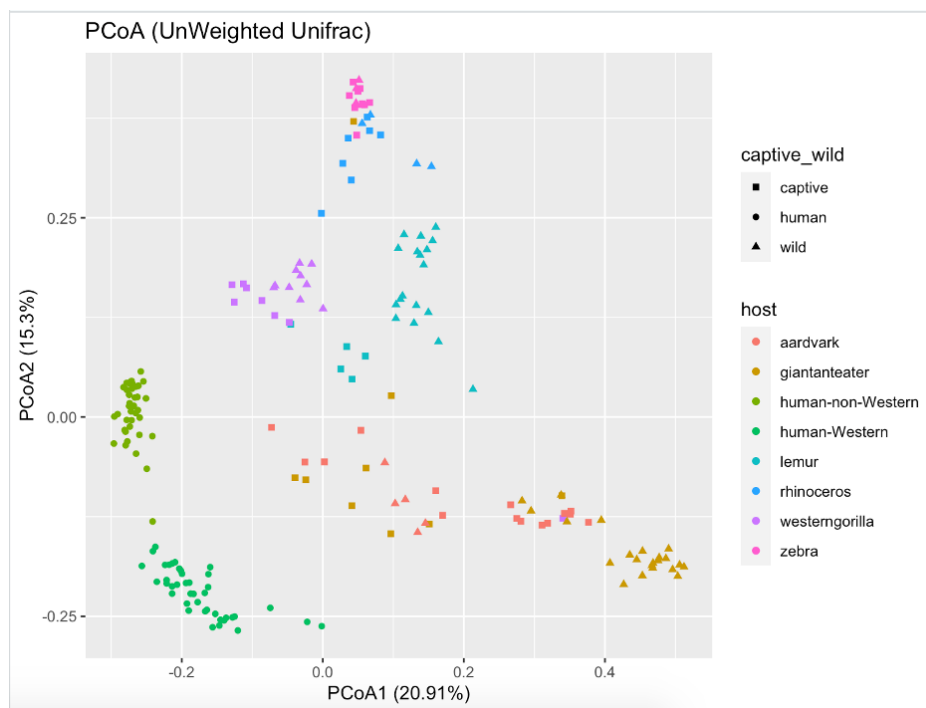
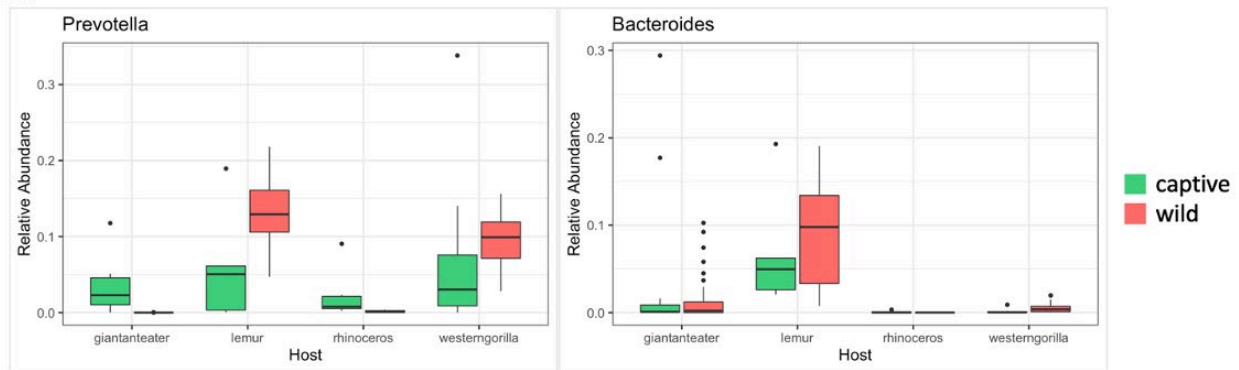


FIG. 2 Captivity Leads to Convergence towards the Human Gut Microbiome. PCoA plot based on unweighted UniFrac distances of gut microbiome of 6 different species of captive and wild animals as well as those of non-Western and Western humans. Convergence towards the human gut microbiome is observed among captive animals in 4 out of 6 species: giant anteaters, lemurs, western gorillas and rhinoceros. Shapes represent captivity status and colors hosts. Refer to Supplementary Figures 1&2 for box plot representation of unweighted UniFrac distances.

Convergence is not driven by *Bacteroides* or *Prevotella*. Next, in order to understand what was driving convergence in these animals, we proceeded to look at the gut taxonomic composition. (As zebras and aardvarks did not show any sign of convergence, they were removed from all further analyses). According to Clayton *et al.*, higher relative abundance of *Bacteroides* and *Prevotella*, the two dominant genera of the human gut microbiome, were observed in captive primates compared to their wild or semi-captive counterparts (9). We therefore hypothesized that we would see similar results with all, if not, at least the primate species. However, to our surprise, we observed lower relative abundance of both *Bacteroides* and *Prevotella* in captive lemurs and western gorillas, with a near-complete absence of *Bacteroides* in the latter (Fig. 3A). Captive giant anteaters and rhinoceros, on the other hand, showed higher relative abundance of *Prevotella* compared to their wild counterparts, although the difference was not statistically significant for rhinoceros ($p=0.235$). This finding indicated that convergence is not driven by *Bacteroides* or *Prevotella*, but rather by other taxon/taxa. Indicator taxa analysis at the genus level (Table S3) revealed the presence of a number of human-associated microbes in captive animals that were either absent or present at very low abundance in wild animals. Among these, *Faecalibacterium*, *Blautia*, *Oscillospira* and *Streptococcus* were identified to be the most differentially abundant (Fig. 3B). Our results

therefore suggest that convergence in captive animals is not driven by dominant bacterial taxa such as *Bacteroides* or *Prevotella* but rather by multiple subdominant taxa.

A



B

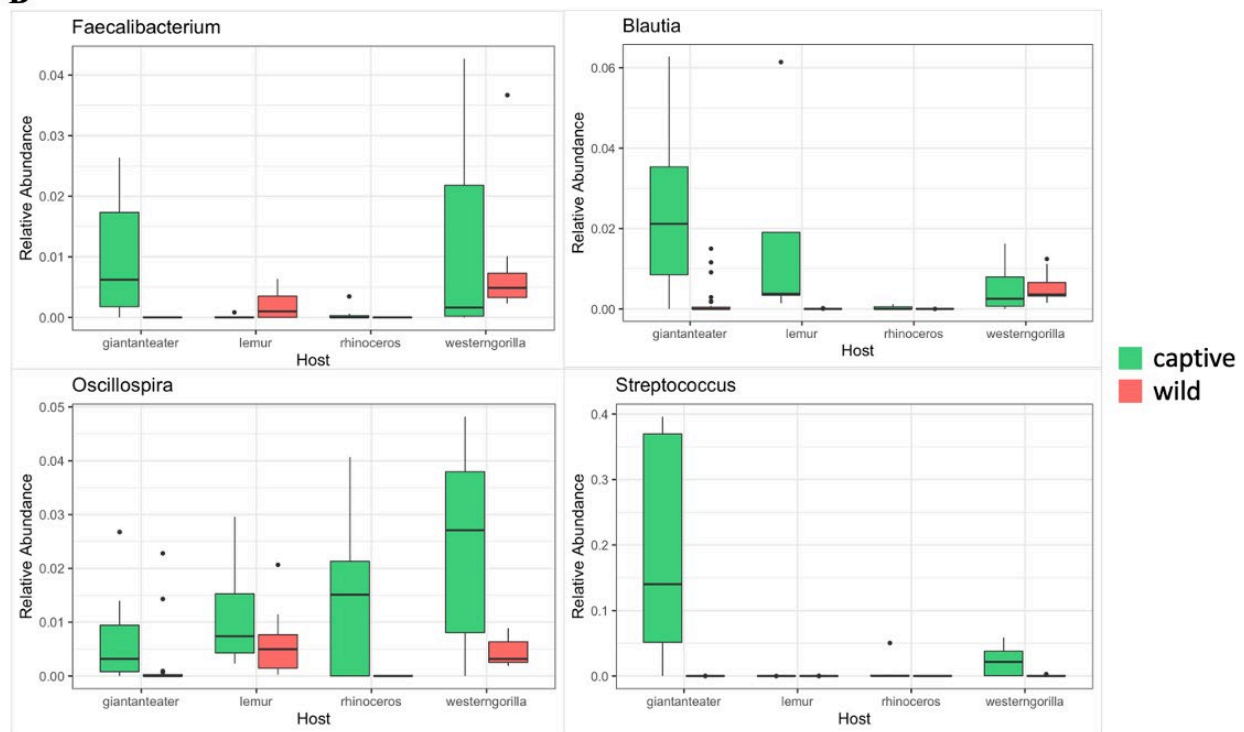


FIG. 3 Multiple subdominant genera responsible for driving convergence in captive animals. Box and whisker plots comparing the relative abundance of (A) *Bacteroides* and *Prevotella*, and (B) *Faecalibacterium*, *Blautia*, *Oscillospira* and *Streptococcus*, in captive and wild animals. *Bacteroides* and *Prevotella*, the dominant genera of the human gut microbiome, are present in lower relative abundance, whereas subdominant genera such as *Faecalibacterium*, *Blautia*, *Oscillospira* and *Streptococcus* are present in higher abundance in captive animals.

Primate microbiota form distinct clusters based on diet composition. Since neither geography nor captive status seem to significantly determine the microbiome of primates, we have decided, for our third and final hypothesis, to investigate the influence of diet on gut microbiota using primates as a case study. For this analysis, we first subsetted the data for primates, which included 145 entries across 22 different species. We observed changes in Shannon alpha diversity across multiple diet compositions, validating the hypothesis of certain food compositions causing decrease in gut microbial diversity. Here we observed a decrease in relative abundance within animals predominantly consuming fruit (Fig. S4). As fruit is the dominant food source for primates in captivity, this could be a potential factor driving the loss of microbial diversity in captive primates. Next, to find out whether different diets lead to shifts in microbial composition, Weighted Unifrac distance metrics were analyzed using Principal coordinate analysis (Fig. 4). We observed 3 distinct diet clusters:

predominantly fruit, a hybrid of fruit and plant, and lastly, diet with some form of animal incorporated including vertebrates or invertebrates collectively form a single cluster. Interestingly, plant-based diet and fruit-based diet formed distinct clusters different from that of the hybrid and were closer to each other than to the hybrid. Moreover, the plant diet demonstrated the greatest heterogeneity as it was not significantly different from any other diet composition other than the plant-fruit hybrid.

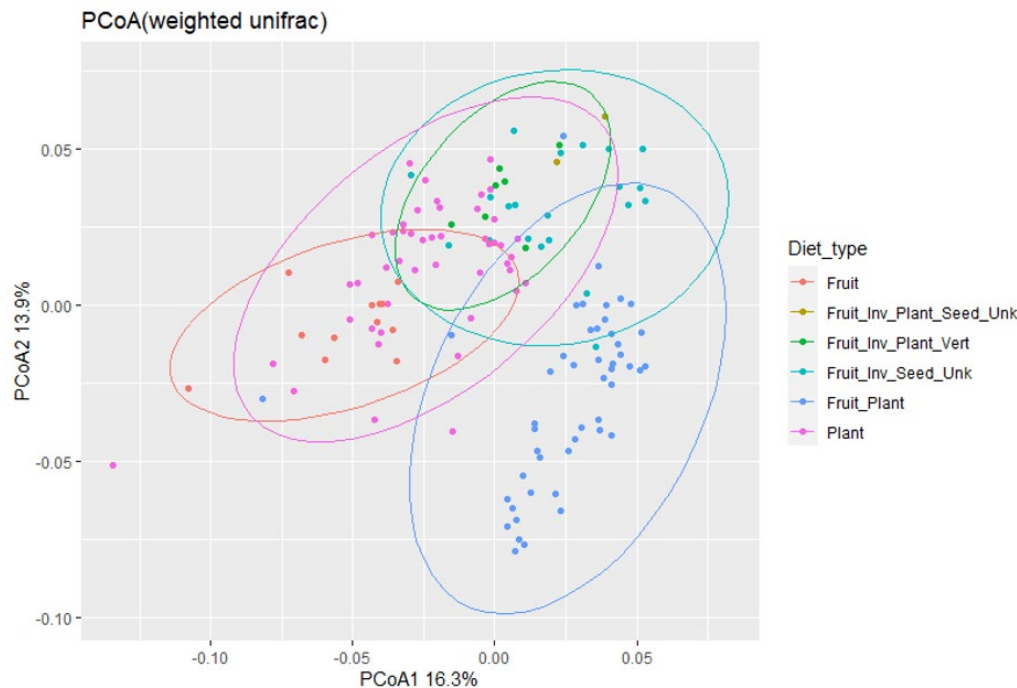


FIG. 4 Weighted UniFrac principal coordinate analysis demonstrates distinct clusters of primate microbiota across similar diet types. The primate microbiota beta diversity distance using weighted UniFrac with respect to diet composition. Food sources refer to Fruit, Plant, Invertebrate (Inv), Seed, Vertebrate (Vert), Seed and Unknown animal (Unk). Circle represents significant clusters of each diet composition.

Certain bacterial classes are associated with each food source. To further investigate the influence of diet on gut microbiota, a set of differentially abundant bacterial classes were assessed for their relative abundance in the two major food sources: plant and fruit (Table S4). Of the differentially abundant classes, 3 bacterial classes, Planctomycetes, Erysielotrichi and Anaerolineae demonstrated some form of significant correlation to at least one of the food sources. Notably, all 3 bacterial classes demonstrated opposite trends of correlation to either fruit or plant. Both Anaerolineae and Planctomycetes were seen to be positively correlated with plant diet, while Erysielotrichi correlates positively with the fruit diet (Fig. 5).

DISCUSSION

Many factors have been related to changes in the gut microbiome (23, 24). To further confirm past research findings and discover new ones, we investigated the influence of geography, captivity, and diet on the mammalian gut microbiome (9, 25–27).

Gut microbiota may converge with similar geography in captive animals. Our PCoA plotting of the different animal subsets suggested that animals living within the same geographic location tended to have similar gut bacterial composition, and that those who resided at different locations are distinctly different. However, the gorillas were the only group that did not exhibit this trend, with Beauval Zoopark samples not clustering, and one sample clustering with the Zoo Atlanta sample (Fig. S1C). In contrast, previous studies have shown that in primates, there is variation in gut microbiota driven by geographical separation (7). This may be due to low sample sizes, with Zoo Atlanta containing only a single sample. Additionally, all of the animals analyzed were captive animals, so there are other factors that might not be applicable to wild animals, such as adaptation to a foreign environment. Overall, it appears that there is a pattern of gut microbiota being similar in animals at a similar geographic location.

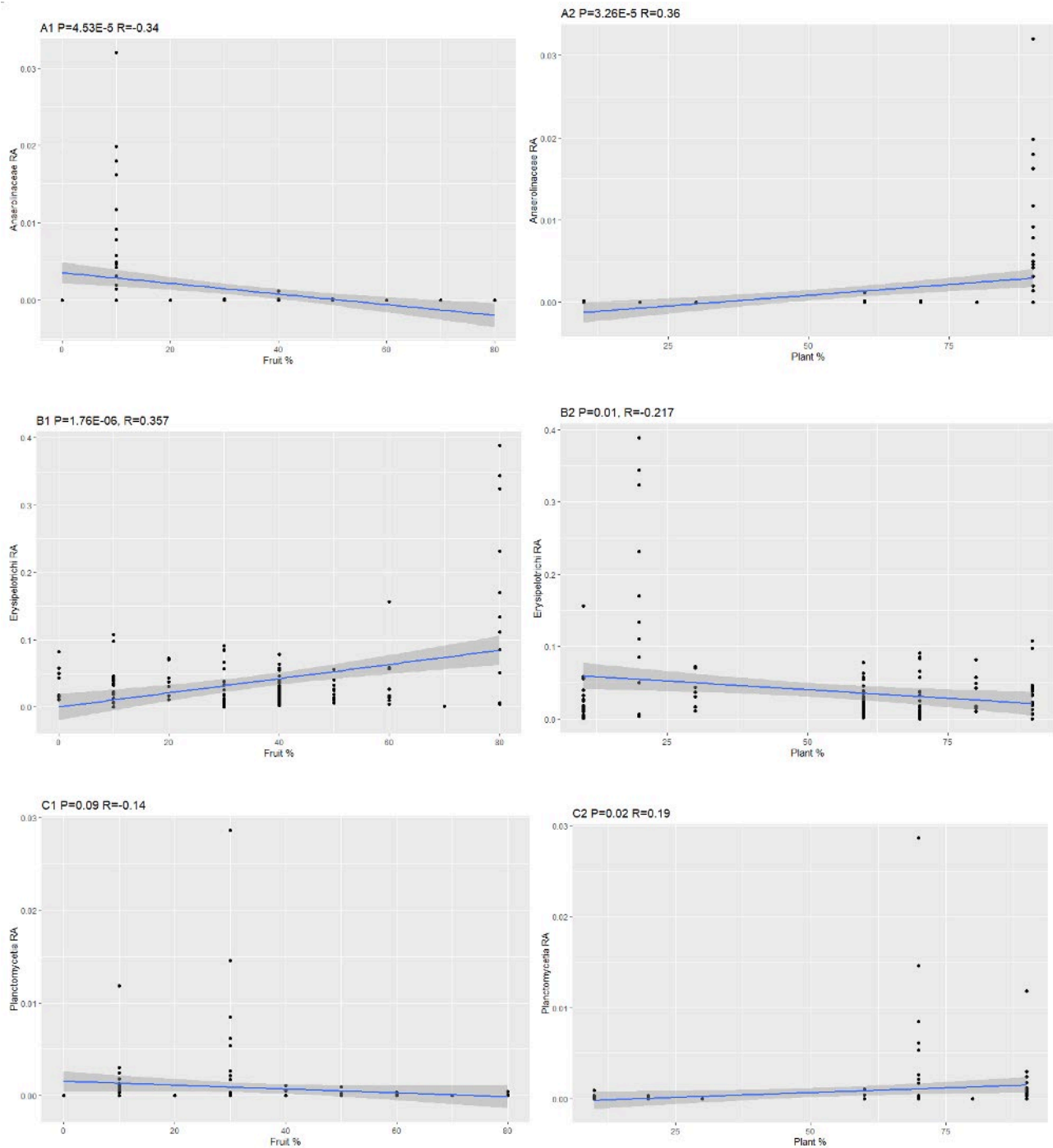


FIG. 5 Bacterial classes show correlation with the proportion of either plant for fruit in their diet. Correlation of top differentially abundant species are analyzed for correlation with either the proportion of fruit or plant in the primate's diet. A = Anaerolineae, B = Erysipelotrichi, C = Planctomycetes. 1 = percentage of fruit, 2 = percentage of plant. Correlation reported in Pearson correlation coefficient.

Diet and environment are interrelated with geography affecting the gut microbiota in myrmecophagous animals. Two groups with relatively large sample sizes at the species-level, giant anteaters and armadillos, are both myrmecophagous animals with highly specialized diets. At different locations, there appears to be differences in diet that are reflected by the indicated taxa. Within the giant anteater group, at National Zoo, the families *Verrucomicrobiaceae* and *Anaeroplasmataceae*, are often found within the microbiome of arthropods (28, 29); at Zurich Zoo, *Leuconostocaceae* are often associated with the surfaces of green vegetation (30), indicating a variation of diet between the two locations (Table S1).

Within the aardvark group, there also appears to be a similar pattern between diet and geography. At Cincinnati Zoo, *Succinivibrionaceae* and *Prevotellaceae* have been associated with starch and plant-rich diets, respectively (31, 32). At Colchester Zoo, bacteria in the identified *Flavobacteriaceae* and *Micrococcaceae* families are often found in arthropods (33, 34). Interestingly, at London Zoo, identified taxa such as *RF16* and *F16* are usually associated with ruminant animals (35–37), perhaps indicating these animals have a more plant-based diet, that require the presence of fermenters. Lastly, at TdR Nature Reserve, instead of indicating taxa that suggests differences in diet, presence of *Alteromonadaceae* and *Geodermatophilaceae* illustrates the presence of an aquatic environment (38, 39), which is reflective of the reserve being situated between two rivers, compared to other locations, which are in more urban environments. Geography allows for factors such as diet and environment to drive gut microbiota into having varied taxa.

Presence of human-associated microbes leads to convergence of animal gut microbiome.

The purpose of the second part of our study was to elucidate the effect of captivity on the humanization of animal gut microbiome. We hypothesized that given the extreme lifestyle changes that captive animals are subject to, from diet restrictions, close interactions with zoo and veterinary staff, to constant exposure to human-built and -maintained environments that a shift towards the human microbiome would occur among captive animals. Our results showed that convergence is not universal, however, as some animals did not show evidence of this pattern. These animals included zebras and aardvarks, the latter of which showed significant variability among the captive population (one group clustered towards the human population while another clustered away from it on the PCoA plot). This could most likely be explained by captive animal samples being collected from different zoo sites, although geography did not seem to affect other animals. Another important finding of this study is that of the animals that did show convergence, not all were seen to converge to the same degree: the western gorillas showed the weakest trend while the giant anteaters showed the strongest. This was highly unusual in that we expected to see a more pronounced shift in primates, as their diet is severely altered in captivity (40). However, this may not have been true for this particular group of gorillas and that other factors were at play, but without the full diet information, it is difficult to make any direct conclusion.

In captive primates, we observed lower relative abundance of *Bacteroides* and *Prevotella* compared to their wild counterparts. *Bacteroides* in humans are associated with animal-based diet, whereas *Prevotella* is associated with plant-based and carbohydrate-based diets (41). The overall reduction of these microbes in captive primates is suggestive of disruptive dietary practices in captivity, with reduced variety of plant and animal-based products in diet. Interestingly, Clayton *et al.* found higher abundance of these taxa in captive red-shanked doucs and mantled howler monkeys. This seemingly contradictory finding may, however, be explained by the fact that red-shanked doucs and mantled howler monkeys are folivores/frugivores, specializing in leaf consumption (42, 43). This type of diet may be hard to mimic in captivity and as such, is often substituted or supplemented with other types of vegetables not found in the wild such as carrots, corn, lettuce and zucchini (44). Lemurs and gorillas, on the other hand, are omnivores, and a restricted diet in captivity could explain the reduction of gut microbial diversity.

Finally, we showed that captive animals became colonized with common human gut microbes that are not present in wild animals. Of these, we identified 4 main genera: *Faecalibacterium*, *Blautia*, *Streptococcus* and *Oscillospira*, which in humans are associated with food degradation and maintenance of intestinal immune homeostasis (45–47). Interestingly, whereas the first three genera were seen to increase only in some captive animals, *Oscillospira* was observed to increase significantly in all four mammalian species. While *Oscillospira* has been positively associated with health in humans (48), the effect of their presence in the animal gut microbiome has yet to be elucidated. Overall, our results point to the conclusion that humanization of captive animal gut microbiome is not universal and that it is driven by multiple subdominant bacterial genera.

Clustering of primate gut microbiota may be influenced by taxonomic relationship as well as diet composition.

Primates across multiple species in the wild and in captivity are

demonstrated to form distinct clusters (Fig. 4), with fruit, animals and hybrid fruit and plant appearing to be the three significant groups. Within most groups we were able to detect a number of different species, especially in the broad carnivorous group, which provide evidence of converging microbiota towards specific diet sources across multiple species. Although this finding is consistent in the plant-fruit hybrid and fruit groups, the results are much more ambiguous in these cases. Firstly, the fruit-based group is heavily dominated by ring-tailed lemur, while a small number of other primates are also clustering within this group, the small sample size may not provide a representative outlook towards the gut microbiota with limited sample sizes. In the fruit-plant hybrid group, all 3 species observed are within the same *Alouatta* genus, or black howler monkey, which may prove to be a more significant influence to the gut microbiota as these species are highly related to one another. Since past studies of various black howler monkey microbiota with regards to its adaptations to environmental changes or different habitat conditions remain mostly on the level of individual species (13, 14), our finding may warrant further investigation of such effects on the level of the whole genus. The surprising finding of primarily plant eaters not forming a distinct cluster may be due to the promotion of fermentation in gut microbiota.

As primates lack the large specialized intestinal compartments for fermentation, adaptations in morphology and enzymes are present in primate digestive tract to compensate. As a result, we may have provided evidence in folivorous primates to adapt to various leafy food sources by possessing a complex fermenting microbiota which may be a subject for further studies and analysis (49–51).

Three bacterial classes found to be significantly correlated with either plant or fruit.

The class Anaerolineae has been identified as a member of anaerobic fermenter in the gut microbiome with significant cellulolytic capacity. Interestingly, the metagenomic analysis of Anaerolineae suggests its reliance on other bacteria to perform the overall cellulose hydrolysis (52). Therefore, our finding is consistent with the past result of plant-based diet inducing the overall population of fermenters. The other class associated with increase in plant within the diet, Planctomycetes has been traditionally thought to be an environmental pathogen and is also identified to be present in the human gut within some populations (53). The past research of Planctomycetes have mostly been centred in extreme environmental conditions including sulfur streams and heavily polluted areas (54, 55). Our findings may suggest a role of Planctomycetes in cellulose breakdown in primates and with the past evidence this may pose implication to human digestion of high fiber diet as well. Erysipelotrichi being the bacterial class associated with high fruit is associated with diabetes, fatty liver and other obesity related markers linked to high sugar intake in humans. However, there is not much research into the class within other animals (56). Our findings would indicate that the fruit being high in sugar would promote such bacteria with metabolism dependent on a high sugar diet and may provide further insight to the health of animals in captivity.

Limitations While we are able to explore some of the potential factors influencing the gut microbiome of various mammalian taxa, sample size was limiting. Many species contained only a few samples (2-4) within the captive and/or the wild subset, which limited the scope of our analysis to very few species. In addition, as a result of subsetting our data, such as from filtering for relevant taxa based on geographical location, the overall statistical power of our analysis was reduced. Furthermore, the lack of detailed characterization of each food source may have led to a dataset not very representative of animal diet in captivity and wild. This also applies to geographical factors; if details including temperature, humidity and other parameters have been included, it would have led to a more detailed analysis

Conclusions In our study, we wanted to determine the impact of geography, captivity and diet on animal gut microbiota. Based on our results, we found that there was a correlation between geography and gut microbiota. When looking into indicator taxa in regards to location, it is shown that there were variations in diet and environment across different locations. We were also able to demonstrate patterns of convergence towards the human gut microbiome in multiple animal species, with the identification of four key taxa driving this

convergence. With primates, we found distinct clusters of gut microbiota based on diet composition and identified 3 classes associated with the dominant food source of the animal.

Future Directions While our study was able to identify some patterns across multiple species, these findings may lack generalizability to other animals that were not included in our study. In addition, in both wild and captivity, changes in food source are difficult to monitor. As such, in future a large-scale multi-species study with well-defined diet information may further elucidate our findings. Studies of wild animals may also be of interest, since a clear understanding of the wild animal gut microbiome may help with improving practices necessary for raising healthy animals in captivity. This may include studies on the variables investigated in the present study, including geography, captivity and overall diet composition.

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