Volume 7: 1-15

The Undergraduate Journal of Experimental Microbiology & Immunology (+Peer Reviewed)

Captive animal gut microbiome is populated with microorganisms that are relevant to the digestion of host dominant diet

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SUMMARY Diet contributes to changes in animal gut microbiomes, which in turn impacts host fitness and immune system development. Drastic changes in diet are often inevitable when animals adjust to captive lifestyles in facilities and could influence host health status by shifting gut microbiome composition. In this study, we evaluated the effects of the dominant diet component on the variation of gut microbiota biodiversity of captive herbivores, omnivores and carnivore species. We performed 16S rRNA gene amplicon sequencing analysis using QIIME2 and R. Alpha diversity differed significantly (Faith's phylogenetic distance $p = 0.04$; Pielou's evenness $p = 0.01$) among herbivore groups primarily consuming fruits versus general plant materials, but not observed for the same diet grouping in omnivores. Captive carnivores with invertebrate-dominant diets had higher phylogenetic diversity ($p = 0.005$) compared to those primarily consuming mammals and birds. The most prevalent and abundant microbial taxa in the gut microbiota vary with the general diet types (carnivore, herbivore, and omnivore). Additionally, among the dominant food-type consumed, the key microbial taxa that significantly differ in abundance likely play functional roles in host digestion. These insights to bacterial biodiversity within captive species, ranging from herbivorous to carnivorous species, can potentially aid conservation management practices that aim to improve animal health and wellbeing in captivity.

INTRODUCTION

The mammalian gut microbiome plays a role in the maintenance of host health, initiation and progression of diseases (1). The microbial community contributes to the fermentation of non-digestible substrates such as dietary fibres, nutrient metabolism and energy acquisition (2, 3). Diet is a key contributor to gut microbiome diversity in animals (4–6). Diet-driven alterations on the mammalian gut microbiota diversity have been observed in multiple studies (4, 7–10) and these changes are becoming increasingly important for understanding host development, immune system and physiological function (4, 11–13). Additionally, captivity in human-constructed environments introduces a series of lifestyle changes that alter the gut microbiota of animals, which may consequently affect host health and disease (2, 11, 14, 15). Factors associated with captive conditions that could influence the gut microbiome include changes in diet, reduced interaction with other species and increased exposure to microbes that thrive in the built environment and human-associated microbes (14).

A major challenge for animal facilities, including zoos and rehabilitation centers, is the maintenance of animal health and well-being (14, 16, 17). In addition to its role in the maintenance of normal development, the gut microbiota also serves as a barrier to pathogens

Published Online: September 2021

Citation: Yu Hsin Chu, Ziwen Ran. 2021. Captive animal gut microbiota is populated with microorganisms that are relevant to the digestion of host dominant diet. UJEMI+ 7:1-15

Editor: Daniela Morales, Stefanie Sternagel and Brianne Newman, University of British Columbia

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(18). Gut microbiome dysbiosis, the alterations in gut microbiota composition that results in functional changes (19), affects the ability to sustain normal physiological functions (13) and is associated with certain diseases in animals (13, 18, 19). Reversing gut microbial dysbiosis with diet has been used as a therapeutic approach for treating disease in felines and its application is being explored for humans (9, 20, 21). Therefore, understanding the effects of diet on the gut microbiota could lead to valuable insights that may promote animal health in fields such as captive breeding programs, veterinary medicine, and conservation of endangered species (14, 16, 17).

Currently, studies on the effects of diet on the composition of mammalian gut microbiomes are limited to few species and relatively small datasets (2, 4, 7, 11, 14, 22). Our study aims to fill this knowledge deficit by analysing a larger dataset containing 41 mammalian species assembled by McKenzie et al (14).

In this study, we examined the variation in gut microbiota with different main dietary habits in captive mammalian species using a dataset collected by McKenzie et al. that includes mammals belonging to six orders and information on their respective diet compositions (14). The original study conducted by McKenzie et al. focused on a comparative analysis between the gut microbiota of captive and wild animals and found varying effects of captivity on microbial diversity (14). This dataset consists of sequenced 16S rRNA gene amplicons from mammalian gut microbiota that could be characterized and used to address our research question on the contribution of diet, namely how dominant dietary components influence the composition of the gut bacterial community (14). Based on previous studies regarding the impacts of diet on gut microbiota, we expect diet to contribute to significant variation in the animal gut microbiota composition. For this study, we utilized 16S rRNA amplicon sequences, collected by McKenzie et al. through amplifying regions of interest on the 16S rRNA gene for analysis of genetic variations in specific genomic regions (23). The resulting sequenced amplicons were processed using QIIME2, a bioinformatics software for microbiome analysis (24) and R for differential abundance analysis of microbial taxa and generation of diversity plots (25).

METHODS AND MATERIALS

Dataset and Metadata Categories. McKenzie et al. collected data from 297 paired wild and captive animals, totaling to 41 mammalian species from six orders, including Carnivora, Cetartiodactyla, Perissodactyla, Primates, Tubulidentata and Pilosa (14). The fecal samples were collected through a global collaborative network from eight different zoos in the USA, France and Switzerland and from wild mammal populations in Central America, South America, South Africa, and Mongolia. DNA was then extracted from the fecal samples, amplified, sequenced, and demultiplexed using QIIME 1.9.1 (14, 24). We based our study on the demultiplexed 16S rRNA gene amplicon sequence data accessed from NCBI Bioproject (PRJEB29017).

The dataset provides a comprehensive set of covariates for each sample, including diet diversity index, diet categories, habitat type, social group sizes, body mass, gut fermentation type, nocturnal or diurnal lifestyle and conservation status. Our study focused solely on diet types and diet categories.

Preliminary Dataset Processing. We imported the demultiplexed sequence data into QIIME2 and performed quality filtering using the Divisive Amplicon Denoising Algorithm 2 (DADA2) (23) where sequences were truncated to 170 nucleotides at a base-call quality cutoff of 25 to ensure adequate sequence quality. The quality filtering step generated a features table and a representative sequences table containing amplicon sequencing variants (ASVs). We choose to generate ASVs instead of operational taxonomic units (OTUs) used by McKenzie et al. because ASVs can be resolved down to single nucleotide differences which allows finer and improved resolution for taxonomic classification and diversity analysis (26).

Following denoising, we constructed a rooted phylogenetic tree based on sequence similarity to infer the evolutionary relationship between microorganisms. We used the

representative sequence artifact generated from denoising and inserted it into the Greengenes (13_8 released) 99% identity reference tree backbone to build a fragment insertion tree with high taxonomic resolution (27, 28). We chose to build a fragment insertion tree instead of a traditional alignment because it provides a more precise phylogenetic reconstruction and increases resolution (29). Taxonomy was assigned using the Naive Bayes classifier trained from the Greengenes 99% database for the alignment of the 16S rRNA gene V4 region (14, 29, 30).

We filtered the dataset to retain only captive animal samples using QIIME2. Then, to control for general diet type when we compare across different dominant diet categories, we created subsets of the samples so that each filtered feature table contains only samples from one general diet type (carnivores, herbivores and omnivores). We conducted our investigation at the animal order level, same as the original study conducted by McKenzie et al. in order to have comparable findings.

Identification of Dominant Diet Categories. We sorted the samples based on the three diet types, carnivores, herbivores and omnivores using the categories provided by McKenzie et al. (Supplementary Table 1). The diet components were categorized based on Eltonian trait diet categories: plant-based categories including fruit, unclassified or general plant materials (plantO), nectar, seed and meat-based categories including invertebrates (Inv), mammals and birds (Vend), scavengers, warm-blooded vertebrates, fish, and unknown vertebrates (31, 32). We identified the dominant diet category as the category that comprises 50% or more of an animal's diet, because it would represent the food type an animal feeds on the most. It is worth noting that the sample sizes for carnivore and omnivore are relatively small, and the sample distribution between different dominant diet categories is uneven in carnivore and herbivore samples (Supplementary Table 2).

Statistical Analysis of Animal Microbiota Diversity. Using QIIME 2, we rarefied to a depth of 70,000 reads per sample to maximize the number of features and samples retained and performed alpha and beta diversity analysis for the feature tables of captive carnivores, herbivores and omnivores respectively. Faith's phylogenetic diversity and Pielou's evenness index were calculated for samples of each diet type to assess microbiota variation within each dominant diet category. The significance of each alpha diversity metric between different dominant diet categories was tested using pairwise Kruskal-Wallis tests. Beta diversity was performed to compare microbial community variation among samples in each diet category using Unweighted and Weighted UniFrac metrics. The significance of beta diversity clustering was tested using the PERMANOVA test.

Differential Abundance Analysis. The outputs generated in QIIME2 (feature table, taxonomic classifications, phylogenetic tree) along with the corresponding metadata, were imported to R (version 4.0.5) and assembled into a combined object using the phyloseq package (version 1.34.0) (25, 33). We excluded low-abundance ASVs that represent less than 0.005% of total sequencing reads from the dataset. We transformed the data to relative abundance in phyloseq for differential abundance analysis between different dietary groups using the DESeq2 package (version 1.30.1) (33, 34). The significance of differential abundance was assessed using adjusted Wald test p-values generated by DESeq2.

Data Visualization in R. Alpha diversity data generated in QIIME2 was imported to R, where alpha diversity box plots were generated using the ggplot2 package (version 3.3.3) (35).

Beta diversity principal coordinates (PCoA) results were generated in QIIME2 and exported to R with the qiime2R package (version 0.99.5) (36). We subsequently generated PCoA ordinations of the beta diversity metrics for captive herbivores, omnivores, and carnivores respectively using ggplot2 (36).

For differential abundance analysis between dominant diet groups, the log2 fold change of microorganism taxa at the class level were plotted using ggplot2 (35). Additionally, we plotted the relative abundance of Fusobacteria in the gut microbiota of captive carnivores as a box plot using the ggplot2 package (35).

Data availability. 16S rRNA sequence data with corresponding metadata is available at NCBI Bioproject (PRJEB29017). QIIME2 processing commands, data filtering and grouping, diversity analysis and taxonomic classifications can be found in the supplementary QIIME2 script. R commands for metadata filtering, grouping and differential abundance are contained in the supplementary R script.

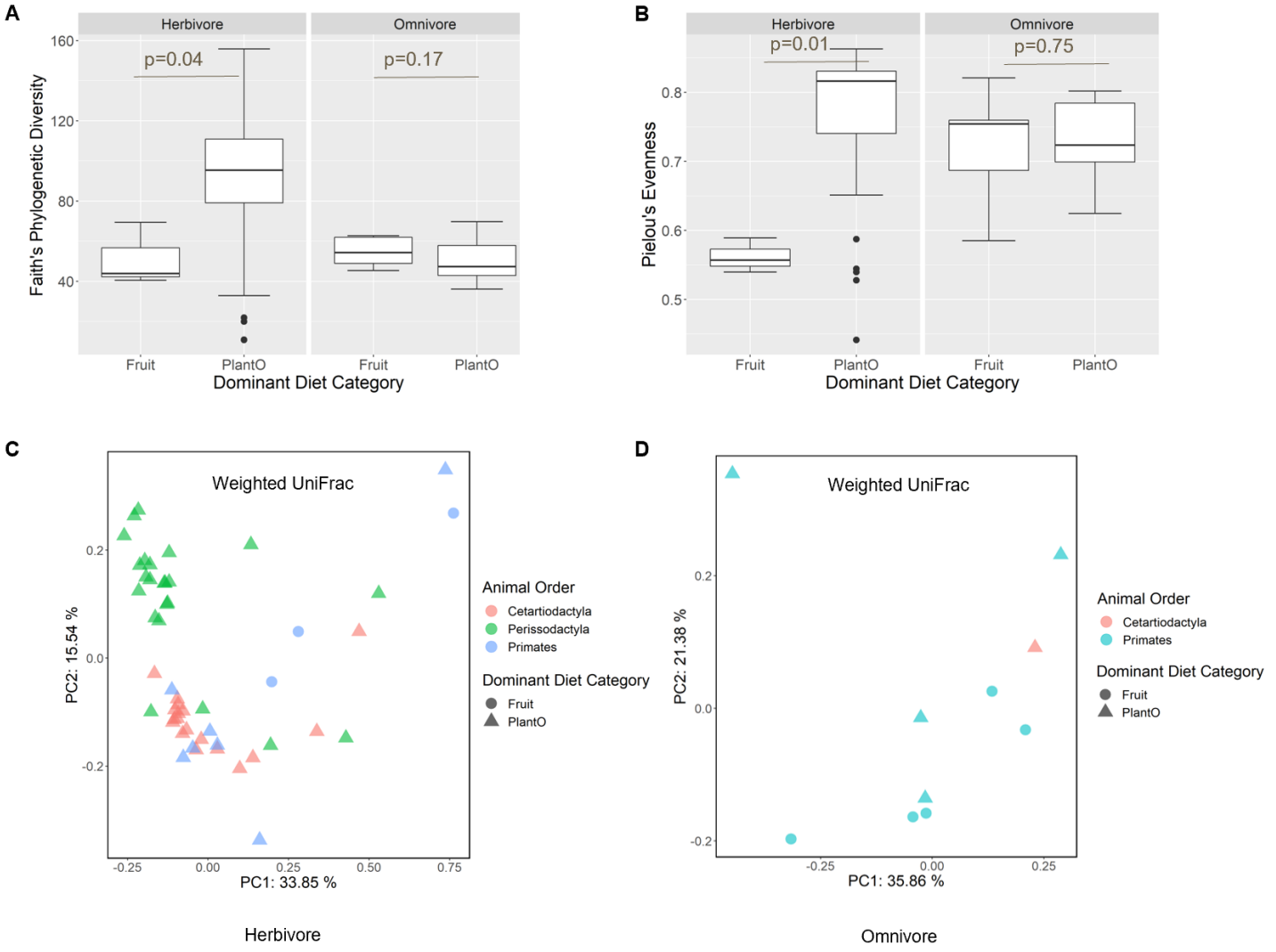

FIG. 1 Dominant diet categories appeared to influence gut microbiota diversity of captive herbivores but not omnivores. Gut microbiota diversity of samples from herbivores and omnivores. (A) Comparison of Faith's phylogenetic diversity measure and (B) Pielou's evenness index for plantO and fruit dietary groups. Kruskal-Wallis p-values are denoted for each comparison within herbivore and omnivore groupings for (A) and (B). (C) Weighted UniFrac PCoA ordination of 16S rRNA gene sequences for herbivore gut microbiota samples. (D) Weighted UniFrac PCoA ordination of 16S rRNA gene sequences for omnivore gut microbiota samples. Samples are coloured by animal order and shaped by dominant diet category for (C) and (D). PlantO: diet consisting of unclassified or general plant materials. Fruit: diet consisting of fruits.

RESULTS

Dominant Diet Category is a Significant Contributor to Alpha Diversity in Herbivores but Not Omnivores. To examine whether different dominant diet categories contribute to gut microbiome diversity in captive animals, we evaluated alpha diversity with Faith's phylogenetic diversity and Pielou's evenness index.

Results showed that for herbivores, there were differences in microbial richness and the relative abundance of microbial taxonomic groups between the gut microbiota of herbivores consuming a plant versus a fruit diet (Fig. 1A, B). Pairwise Kruskal-Wallis tests present a p-value of 0.04 for Faith's phylogenetic distance and 0.01 for Pielou's evenness. Herbivores consuming a fruit-dominant diet showed lower diversity and thus more related microbial communities compared to animals consuming a plant-dominant diet (Fig. 1A, B).

For omnivores, there were no significant differences in microbial evenness or richness ($p = 0.17$ for Faith's phylogenetic distance; $p = 0.75$ for Pielou's evenness) in the gut microbiota based on dominant diet categories (Fig. 1A, B). The gut microbiota composition of omnivores consuming a fruit dominant diet did not differ significantly from those consuming a plant dominant diet. These results suggest that dominant diet categories contributed to variation in gut microbiota diversity in herbivores but not omnivores.

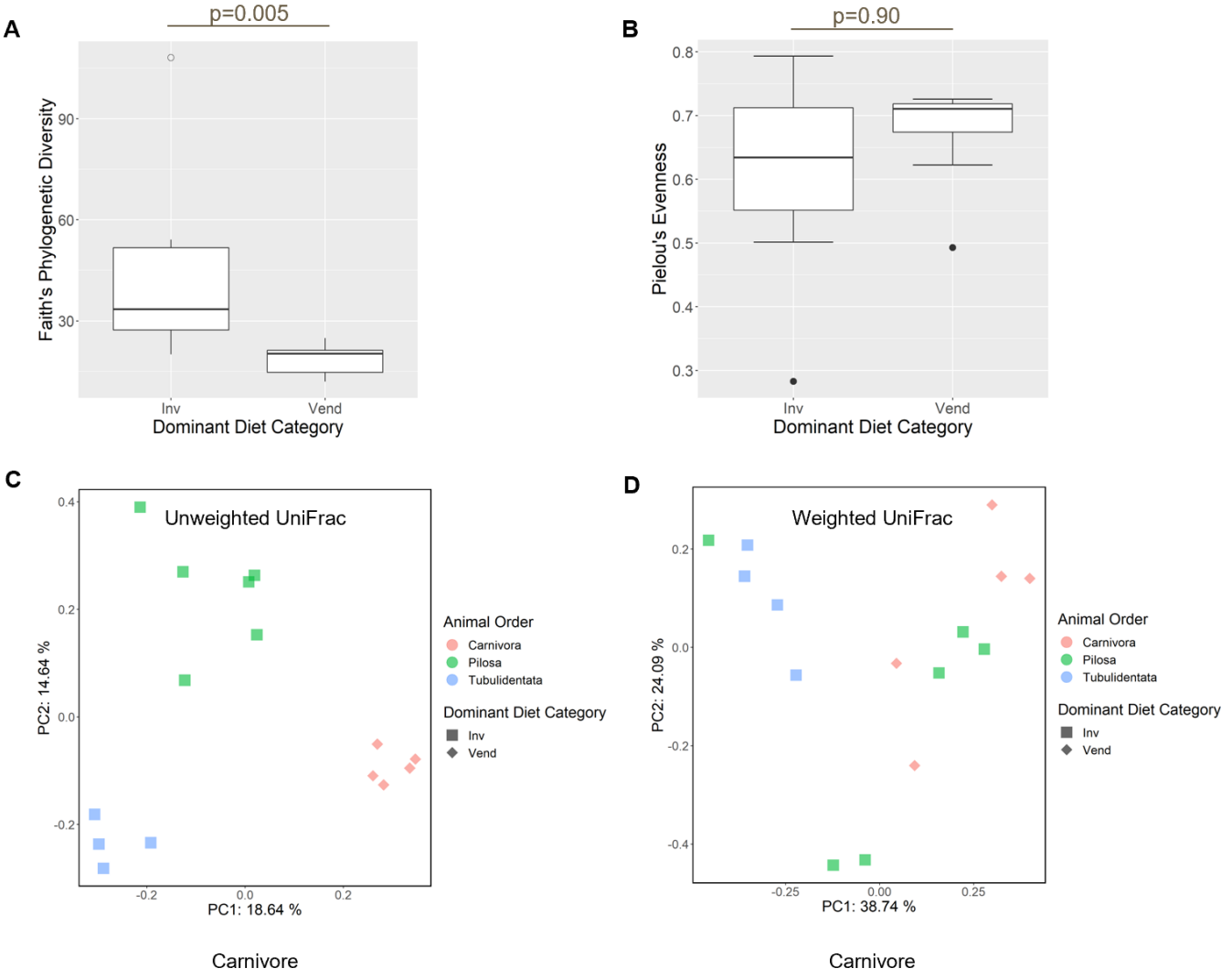

FIG. 2 Gut microbiota diversity of captive carnivores were influenced by dominant diet categories. Gut microbiota diversity of samples from carnivores. (A) Comparison of Faith's phylogenetic diversity measure and (B) Pielou's evenness index for Inv and Vend dietary groups. Kruskal-Wallis p-values are denoted for each comparison within carnivore groupings for (A) and (B). (C) Unweighted UniFrac and (D) Weighted UniFrac PCoA ordination of 16S rRNA gene sequences for carnivore gut microbiota samples. Samples are coloured by animal order and shaped by dominant diet category for (C) and (D). Inv: diet consisting of invertebrates. Vend: diet consisting of mammals and birds.

Dominant Diet Category Is not a Significant Contributor to Beta Diversity in Herbivores or Omnivores. To further investigate the role of diet on gut microbiome composition in animals consuming different dominant diets, we evaluated beta diversity using Unweighted UniFrac and Weighted UniFrac metrics.

We observed no distinctive clustering by dominant diet category or by host phylogeny from the PCoA ordination with Weighted UniFrac for herbivore samples (Fig. 1C), suggesting similar gut microbial communities among herbivores consuming different diets and across host identity. However, statistical significance ($p = 0.006$ for dominant diet category; $p = 0.001$ for host phylogeny) was observed for both factors in PERMANOVA tests. This is likely due to the uneven sample distribution in the herbivore group which contains a limited number of fruit-consuming samples (Supplementary Table 2). For omnivores, we observed no clustering based on dominant diet categories or host phylogeny in the Weighted UniFrac PCoA ordination, indicating that gut microbial richness, evenness and phylogenetic distance were similar among omnivore samples (Fig. 1D). This is further validated by PERMANOVA test p-values ($p = 0.284$ for dominant diet category; $p = 0.391$ for host phylogeny). Together, these results showed that the gut microbiome composition of omnivores were not influenced by their main food type consumed and host identity.

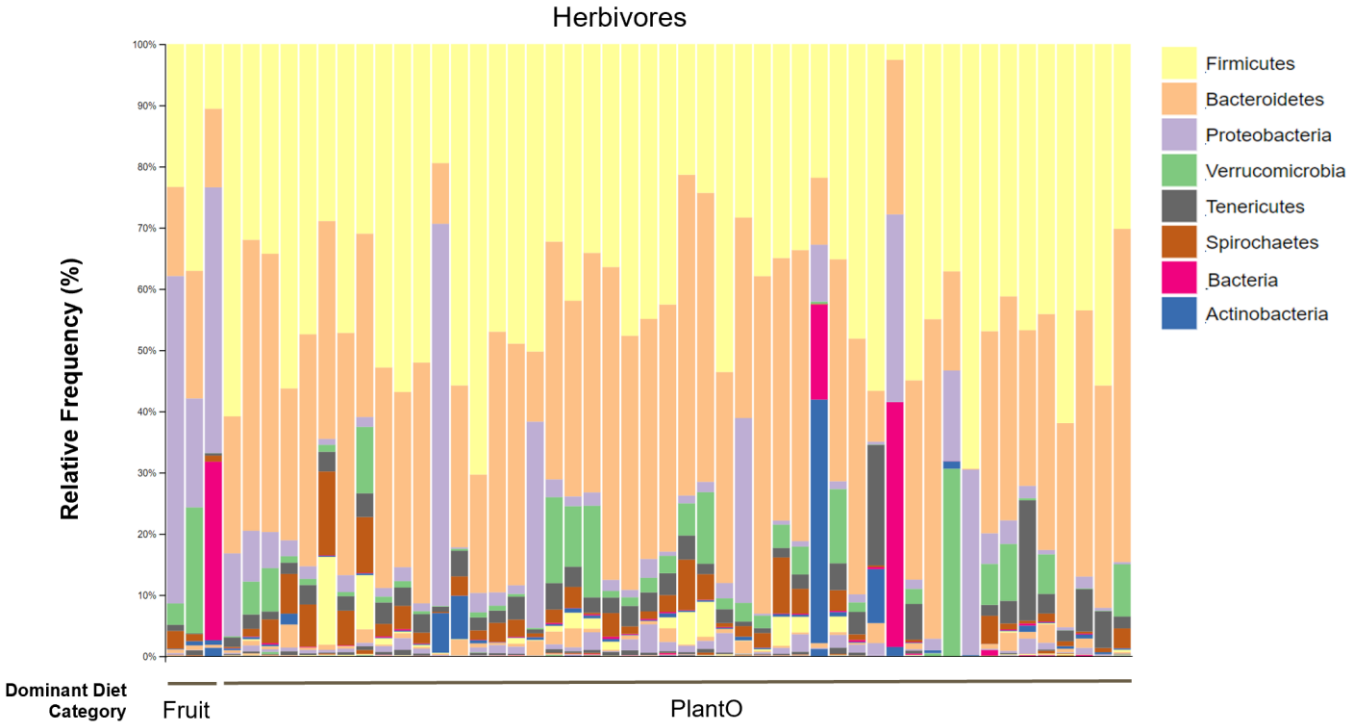

FIG. 3 Taxonomy profile of captive herbivores with fruit and plant as dominant diet categories. Relative frequency of microbial taxonomy groups at the phylum level in each herbivore sample. PlantO: diet consisting of unclassified or general plant materials. Fruit: diet consisting of fruits.

Dominant Diet Category is a Significant Contributor to Alpha and Beta Diversity in Carnivores. To explore the relationship between diet and gut microbial community composition in captive carnivores, we evaluated both alpha and beta diversity using Faith's phylogenetic diversity, Pielou's evenness index, Unweighted UniFrac, and Weighted UniFrac metrics.

There were significant differences ($p = 0.005$) in alpha diversity of the gut microbiota of carnivores based on dominant diet categories when evaluated by Faith's phylogenetic distance (Fig. 2A), but not Pielou's evenness ($p = 0.9$) (Fig. 2B).

Carnivore gut microbial communities clustered by two diet categories ($p = 0.001$), invertebrates (animals consuming ants and termites; Inv) and vertebrates (mammals and

birds; Vend) and by host phylogeny (p = 0.001) using Unweighted UniFrac (Fig. 2C). There was also distinctive clustering of gut microbiome diversity by the three animal orders (Fig. 2C). Together, these results suggest that carnivores consuming the two different diets had gut microbiota composition that differed in phylogenetic distance and richness. Interestingly, when also considering the relative abundance, we saw no distinctive clustering of Weighted UniFrac ordination by dominant diet categories (Fig. 2D) but a p-value of 0.012 from the PERMANOVA test. This can probably be attributed to the small sample size of the carnivore group and the uneven sample distribution between the different dominant diet categories (Supplementary Table 2). Weighted UniFrac displayed some clustering by animal order Carnivora and Tublidentata but not Pilosa by the first axis (Fig. 2D). A p-value of 0.006 lends support to this observation. Overall, dominant diet categories contributed to the variation in phylogenetic distance and microbial richness in gut microbiome diversity in carnivores.

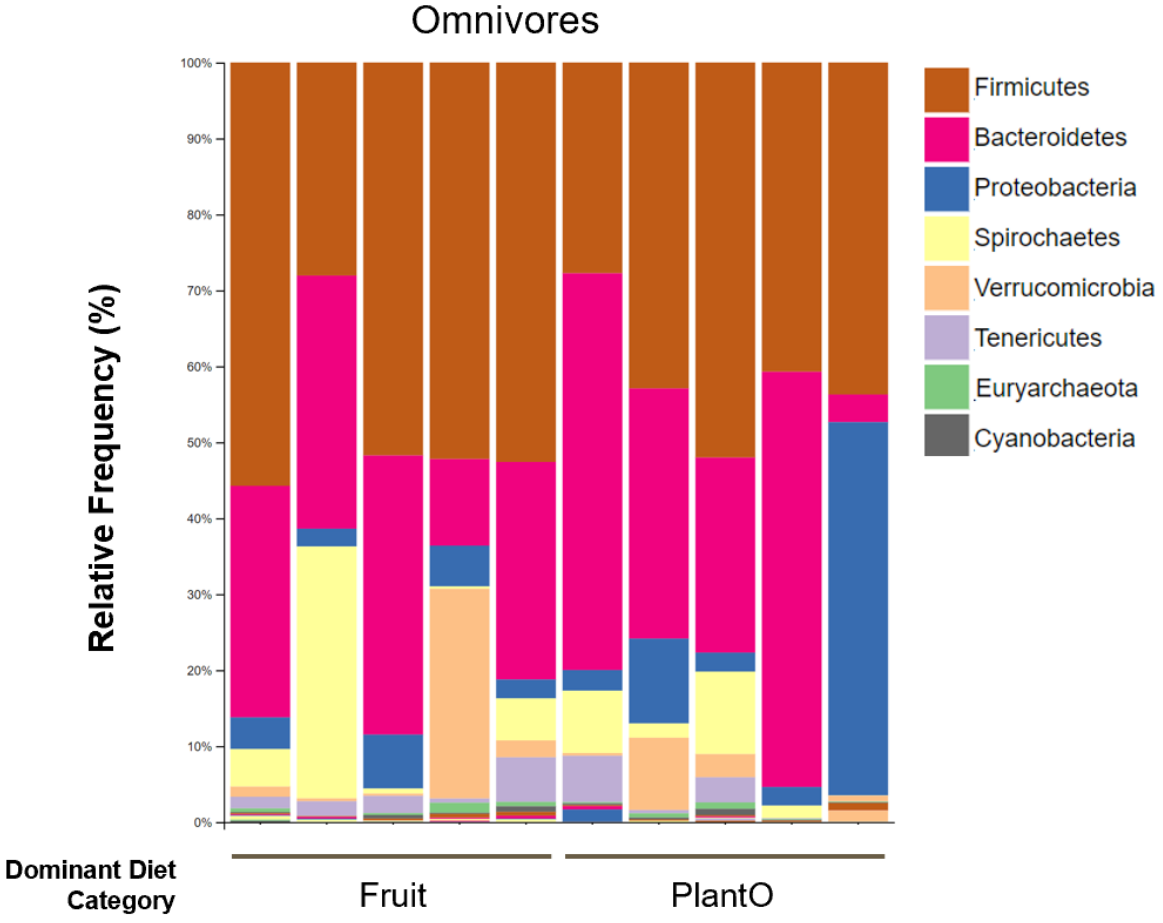

FIG. 4 Taxonomy profile of captive omnivores with fruit and plant as dominant diet categories. Relative frequency of microbial taxonomy groups at the phylum level in each omnivore sample. PlantO: diet consisting of unclassified or general plant materials. Fruit: diet consisting of fruits.

Class-level Variations are Present in the Taxonomy Profile of the Gut Microbiota of Herbivores and Omnivores with Different Dominant Diets. To explore the taxonomy profile of the gut microbiota of captive herbivores and omnivores consuming different dominant diet categories, we examined taxonomy bar plots and performed differential abundance analysis (Fig. 3, 4, 6). Firmicutes, Bacteroidetes, Proteobacteria were prevalent in the gut microbiota of all captive herbivores and omnivores (Fig. 3, 4). Verrucomicrobia was present in most captive herbivore and omnivore samples with varying relative abundance (Fig. 3, 4).

In most herbivores primarily consuming plant material within the plantO category, Firmicutes and Bacteroidetes were the most abundant gut microbiota members (Fig. 3). In the gut microbiota of herbivores with fruit-dominant diets, the most abundant phylum was Proteobacteria (Fig. 3). Fibrobacteria, Mollicutes, Deltaproteobacteria, Fusobacteriia, Cyanobacteria 4C0d-2, Methanomicrobia, Verrucomicrobia Subdivision 5 (Verruco-5), and Sphingobacteriia were significantly more abundant in animals primarily consuming plantO material (Fig. 5). However, the small sample size of herbivores consuming fruit as their main diet compared to plantO-consuming herbivores (Supplementary Table 2) likely affected our statistical analysis. As a consequence, these findings would require further validation with larger and more evenly distributed sample sizes.

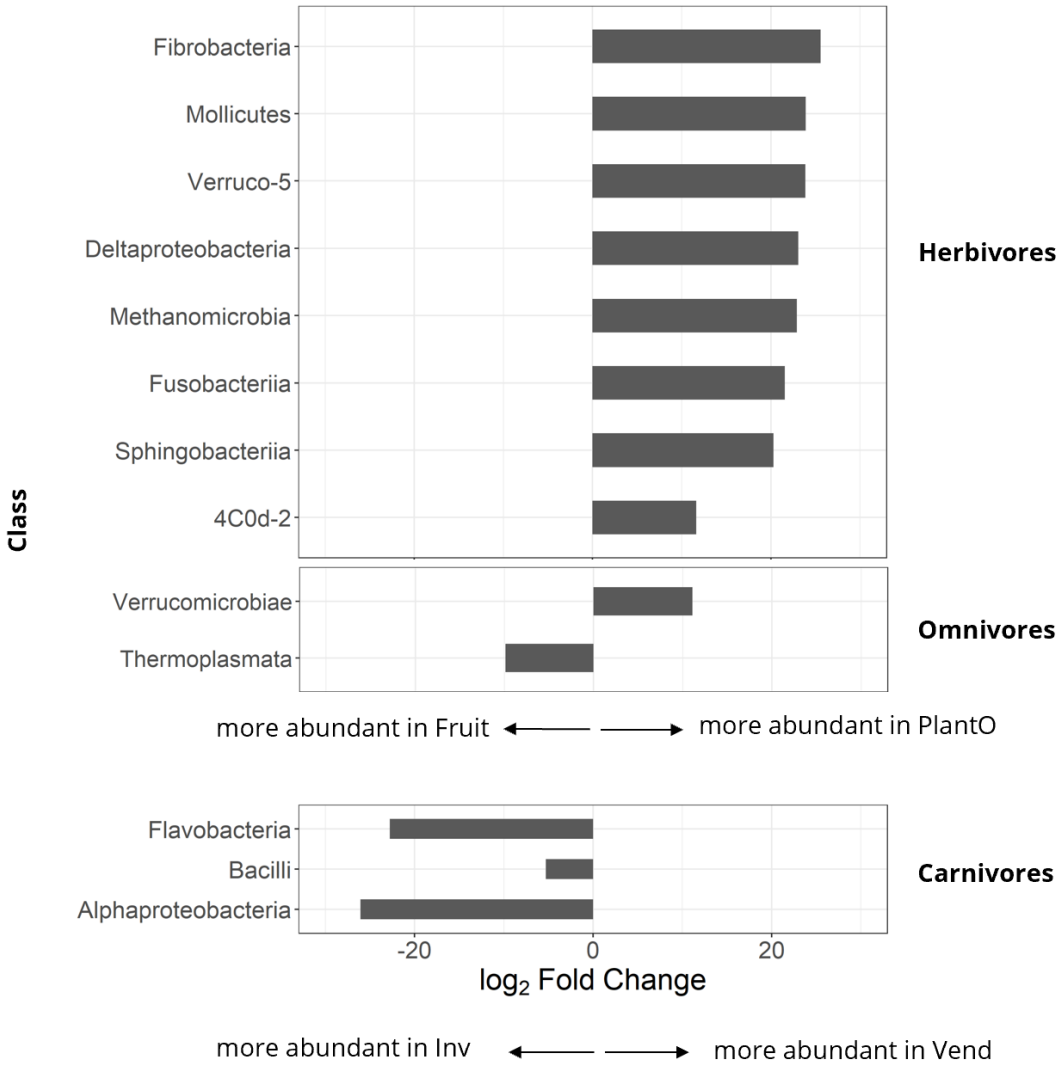

FIG. 5 Differential abundance profile of captive herbivores, omnivores and carnivores at the class level. Evaluation of the relative abundance of microbial taxa in different dominant diet categories using differential analysis. The bars represent log2 fold change. Only microbial taxa with significant fold changes ($p < 0.05$) at the class level are shown. PlantO: diet consisting of unclassified or general plant materials. Fruit: diet consisting of fruits. Inv: diet consisting of invertebrates. Vend: diet consisting of mammals and birds.

In captive omnivores, Firmicutes was the most abundant bacteria phylum for animals consuming either dominant diet categories (Fig. 4). The archaea class Thermoplasmata was significantly more abundant in animals with fruit-dominant diets, while the bacteria class Verrumicrobiae is more abundant in samples with plantO-dominant diets (Fig. 5).

Dominant Diet Categories Impacts the Taxonomy Profile of Carnivore Gut Microbiota at the Class Level. To assess the effect of dominant diet categories on the taxonomy profile of the gut microbiome of captive carnivores, we generated a taxonomy bar plot (Fig. 6A). Firmicutes, Proteobacteria, and Bacteroidetes were prevalent in all carnivore samples, with Firmicutes being the most dominant bacterial phyla in the majority of samples (Fig. 6A). Fusobacteria was more common and more abundant in carnivores primarily consuming mammals and birds compared to those primarily consuming invertebrates (Fig. 6A).

To quantitatively assess the taxonomy composition of carnivore gut microbiome and the effect of dominant diet categories, we calculated the relative abundance of ASVs and performed differential abundance analysis. In the gut microbiome of captive carnivores, the bacteria classes Flavobacteria, Bacilli, Alphaproteobacteria were significantly more abundant in carnivores with invertebrate-dominant diets, where the absolute log2 fold change of Flavobacteria and Alphaproteobacteria exceeds 20 (Fig. 5).

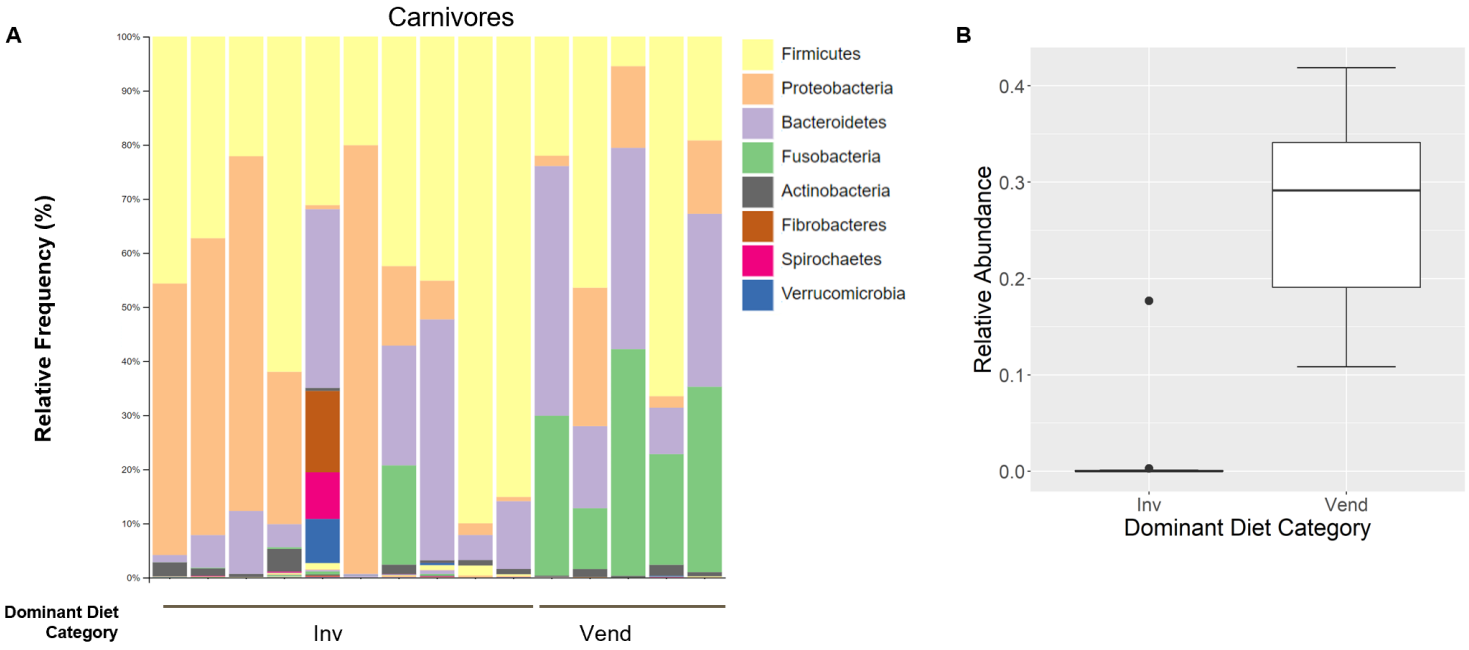

FIG. 6 Taxonomy profile of captive carnivores with invertebrates and vertebrates as dominant diet categories. Relative frequency of microbial taxonomy groups at the phylum level in each carnivore sample (A). Evaluation of the relative abundance of Fusobacteria in each dominant diet category (B). Inv: diet consisting of invertebrates. Vend: diet consisting of mammals and birds.

Although Fusobacteria appeared to be more abundant in one dominant diet category based on taxonomy profile (Fig. 6A), this result was not significant upon evaluation with differential abundance. Therefore, we examined the relative abundance of Fusobacteria in the gut microbiota of carnivores consuming different dominant diet categories (Fig. 6B). Fusobacteria was absent in most carnivore samples with an invertebrate-dominant diet except for several outliers (Fig. 6A, B). On the other hand, although Fusobacteria was present in the gut microbiota of all five captive carnivores that predominantly consume mammals and birds, its relative abundance among samples was highly varied (Fig. 6A, B). In addition, classes within the Fusobacteria phylum did not differ in relative abundance between the vertebrate- and invertebrate-consuming groups.

DISCUSSION

In this study, we examined the 16S rRNA gene amplicon dataset for captive animal fecal samples collected by McKenzie et al. We investigated different animal diet types (carnivore, herbivore, and omnivore) (14), and identified the dominant diet category of each animal as

the Eltonian traits diet category that makes up over 50% of its diet. To explore the effect of dominant diet components on the gut microbiota diversity and composition of captive animals, we performed alpha and beta diversity analysis, taxonomy classification, and differential abundance analysis.

The Dominant Diet Component Affects Gut Microbiota Diversity in Herbivores and Omnivores but is not a Significant Predictor. Our alpha diversity analysis indicated that Faith's phylogenetic diversity and Pielou's evenness varies significantly with the dominant diet categories in captive herbivores (Fig. 1A, B). This suggests the consumption of fruit material or plantO material significantly contributes to relative abundance, richness, and diversity in phylogenetic distance within the gut microbiota of captive herbivores. Herbivores with fruit dominant diets displayed more even and more closely related gut microbial communities (Fig. 1A, B). We also witnessed less variability in alpha diversity metrics for primarily fruit-eating herbivores (Fig.1A, B). Additionally, we found no differences in beta diversity in herbivores according to the Unweighted PCoA ordination (Fig. 1C) but significant p-values when examining the effects of dominant diet and host phylogeny. These observations are likely attributed by the limited fruit sample numbers and uneven sample size in the plantO and fruit categories (Supplementary Table 2). The results for both the alpha and beta diversity in herbivores would need to be validated by a larger and more evenly distributed sample size.

However, we did not witness similar differentiation of alpha diversity by dominant diet categories in captive omnivores consuming plant-dominant diets (Fig. 1A, B). This is likely because the vast majority of omnivorous animals included in this study are non-human primates, whereas samples from a much wider range of species are included in the herbivorous group. We found no differences in beta diversity by dominant diet categories and host phylogeny for omnivores (Fig. 1D). This suggests that neither the dominant diet component nor host identity are significant predictors of the gut microbiota diversity in omnivores primarily consuming plants (fruit or plantO diet).

Herbivore and Omnivore Gut Microbiota are Populated with Microbes that Potentially Aid the Digestion of Their Dominant Diet. Firmicutes and Bacteroidetes were the most abundant phyla in all captive animals, but especially in captive herbivores and omnivores (Fig 3, 4). The predominant presence of Firmicutes and Bacteroidetes in the gut microbiota of herbivores is consistent with findings in multiple previous studies (4, 11, 22).

Captive herbivores with plantO-dominant diets showed higher abundance of eight microbial classes compared to those primarily consuming fruits, including the Fibrobacteres class Fibrobacteria and the Proteobacteria class Deltaproteobacteria (Fig. 5). A similar study found that Fibrobacteres and Proteobacteria are commonly detected in captive herbivorous deer consuming leaves, branches and plants (11). Certain species of Fibrobacteria, such as *Fibrobacter succinogenes*, are fibrolytic and could aid host digestion of high fibre content such as plants and hay (10, 11). This may explain the higher abundance of Fibrobacteria in captive herbivores primarily consuming plants. However, our finding needs to be further validated in future studies as the uneven sample distribution between herbivores consuming primarily plantO and fruit likely affected the results.

For captive omnivores, a significantly higher abundance of the class Verrucomicrobiae was associated with the plantO but not the fruit dominant diet category (Fig. 5). A previous study on the human microbiome has reported a similar pattern with this class of bacteria, showing that a higher abundance of Verrucomicrobiae is associated with vegan diets which generally contain more plant materials compared to omnivore diets (37).

The Dominant Diet Affects Diversity in Phylogenetic Distance but not Relative Abundance of Microbial Groups in Carnivores. Dominant diet category significantly affected Faith's phylogenetic diversity but not Pielou's evenness of the gut microbiota of captive carnivores (Fig. 2A, B). Carnivores consuming a primarily invertebrate-based diet had greater microbial richness/diversity than carnivores consuming mammals and birds. Both groups exhibited similar relative community evenness, which is a measure of the ecosystem balance. This suggests that taxa within gut microbial communities are relatively balanced for

both diet groups. Gut microbial communities of captive carnivores clustered by dominant diet categories and by phylogeny in the Unweighted UniFrac PCoA ordination (Fig. 2C), suggesting that both factors are significant predictors of carnivore gut microbiota composition when considering taxa with lower abundance. Similar findings were reported by a previous study comparing myrmecophages (animals consuming ants and termites) and vertebrate-consuming carnivores (24). When considering the community structure using Weighted UniFrac, clustering by Carnivora and Tublidentata but not Pilosa by the first axis is witnessed, suggesting that the variation in gut microbiota diversity in Pilosa can probably be attributed to less abundant microbial groups (Fig. 2D) .

Bacteria Related to Digestion and Nutritional Supplementation Shows Significantly Different Abundance Between Different Dominant Diets in Carnivores. We assigned taxonomy classifications to ASVs found in carnivore microbiota and performed differential abundance analysis to explore the effect of dominant diet categories on the gut microbiota taxonomy profile (Fig. 5, 6A). The most prevalent and abundant phyla (Firmicutes and Bacteroidetes) (Fig. 6A) are consistent with previous observations in many carnivorous mammalian gut microbiomes such as anteaters, aardvark, dogs, dholes and omnivorous mammals including humans and mice (38–43).

Fusobacteria appeared to be more prevalent and more abundant in carnivores consuming general vertebrates including mammals and birds (Fig. 6A). However, upon differential abundance analysis, we found no significant difference in its relative abundance between the different dominant diet categories. Class-level analysis also did not reveal any significant difference in the relative abundance of any Fusobacteria class between invertebrate- and vertebrate-consuming carnivores (Fig. 5). This lack of statistical significance is probably due to the small sample size of the carnivore group, the occurrence of outliers and the high variability of the relative abundance of Fusobacteria in carnivore gut microbiota (Fig. 6B). The variation of Fusobacteria between carnivores consuming different dominant diets requires further investigation with a larger sample size.

Carnivores primarily consuming invertebrates showed significantly higher abundance of Flavobacteria, Bacilli and Alphaproteobacteria compared to those consuming mostly mammals and birds (Fig. 5). Among these bacteria classes, Flavobacteria belong to the phylum Bacteroidetes, which has been shown to comprise a large portion of the mammalian gut microbiome (16). Bacteroidetes play an important role in host food digestion by breaking dietary polysaccharides into short-chain fatty acids that can be absorbed as energy sources and degrading otherwise indigestible complexes (16). Interestingly, a study on captive cheetahs found an under-representation of Bacteroidetes (16), which mirrors the lower abundance of Flavobacteria in carnivores primarily consuming mammals and birds compared to carnivores consuming invertebrates in our study (Fig. 5).

Limitations Our study was limited by the small sample size for each species. Some captive animals in this dataset contained only one sample, such as cheetah and wild dog (14). Chloroplast and mitochondria sequences were not filtered out of the dataset. These sequences are considered noise in the dataset and may have affected our findings. Additionally, the sample size of the carnivore and omnivore groups are relatively small (Supplementary Table 2). There is also an uneven distribution of samples between different dominant diet categories in herbivores and carnivores (Supplementary Table 2). These limitations in the sample size likely played significant roles in our statistical analysis and potentially skewed our findings. Therefore, our conclusions have limited generalizability and require further validation in larger datasets. Furthermore, the samples in this dataset were collected from a variety of regions across the globe (14). Thus, the gut microbiota of animals could potentially be affected by region-specific pathogens, which are not taken into the consideration of this study

Conclusions Our study aimed to investigate the effect of diet on the variation of gut microbiota diversity and composition of captive animals, specifically how these two parameters vary by the dominant diet categories. Overall, we found that dominant diet category is a significant contributor to gut microbiota diversity in carnivores but not in omnivores, and only to alpha diversity in herbivores. Host phylogeny affects beta diversity in

carnivores but not in omnivores. Upon taxonomic classification, we found that the most prevalent and abundant microbial taxa in the gut microbiota vary with the general diet types (carnivore, herbivore, and omnivore). Gut microbiota members that are differentially abundant in animals with different dominant diet categories appear to be relevant to host digestion. Due to our study limitations, our findings would need to be compared with studies using a greater and more evenly distributed sample size with a higher variety. Nevertheless, our research provides a basis for further studies which address these concerns and aid in determining the impact of animal diets on the gut microbiota health of captive animals and could have important significance for a range of disciplines from veterinary medicine to captive breeding efforts for biological conservation.

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Future Directions Our study investigated the effect of diet by identifying the dominant diet category, which refers to the Eltonian trait diet category (31, 32) that makes up over 50% of the diet of an animal. Using this criterion, we managed to characterize the diet of mammals in the dataset collected by McKenzie et al. (14) and were able to observe trends associated with the dominant diet in the gut microbiota diversity and taxonomy profile of captive animals (Fig. 1-6). Therefore, our study has demonstrated the identification of the dominant diet category as an effective and relevant method for characterizing the diets of animal subjects. Future studies investigating diet-related topics in animals may consider applying our methodology of dominant diet identification to examine the role of the major diet component. The findings of our study were limited by the small and unevenly distributed sample sizes. Hence, future studies could perform a similar analysis pipeline using datasets with more biological replicates and more evenly distributed samples to confirm the trends of the animal gut microbiota with different dominant diet components.

Moreover, the horizon of similar analysis could be expanded to the effect of lesser diet components and dietary supplements. These components may comprise smaller proportions of an animal's diet, but could also contribute to their nutritional supplementation and health maintenance. For instance, fruit is an important dietary source of vitamin C (44) and the lack of vitamin C intake could result in cutaneous petechiae, ecchymoses, hematomas, ulcerations and other vitamin C deficiency symptoms as observed in guinea pigs (45, 46). Another example pertains to linoleic acid and arachidonic acid, which are small but essential nutritional supplements that prevent fatty acid deficiency in cats (45). Therefore, understanding the effect of minor diet components and dietary supplements on animal gut microbiota diversity and composition as well as the health consequences of these effects may also be important.

Lastly, future studies could aim at developing feeding schemes for captive animals that promote healthy microbiota compositions or help animals cope with diseases. In a previous study, Naarden et al. demonstrated that a therapeutic urinary stress diet significantly lowered the rate of feline idiopathic cystitis recurrence (20). Similarly, a diet composition designed to promote the cultivation of beneficial microorganisms in animals' gut could potentially serve as a treatment for gastrointestinal disorders or a therapeutic complement to drugs and thus improve the health and well-being of captive animals.

ACKNOWLEDGEMENTS

We would like to thank McKenzie et al. for providing the dataset that was used in this study. We also want to express our gratitude to the MICB421 course director Dr. David Oliver, the MICB 421 teaching team: Dr. Evelyn Sun, Dr. Stephan Koenig, Zakhar Krekhno, Emily Adamczyk and Mihai Cirstea for their guidance, support and feedback throughout this project. Additionally, we want to thank the Department of Microbiology and Immunology at the University of British Columbia for providing the resources and funding that make this project possible. Lastly, we want to thank Helen Hsiao for her help in running the commands for diversity analysis and taxonomy analysis. We would also like to thank two anonymous reviewers for constructive feedback on this manuscript and our editors Daniela Morales, Stefanie Sternagel and Brianne Newman for their help throughout the peer-review and publication process."

CONTRIBUTIONS

Yu Hsin and Jo collaboratively worked on the QIIME2 workflow in processing and analyzing the data. Jo is responsible for the differential abundance analysis in R.
For writing this manuscript, Yu Hsin is responsible for the introduction section and methods and materials for the QIIME2 workflow and results for diversity analysis. Jo is responsible for methods and materials for the R workflow, results for taxonomy and differential abundance analysis and the future direction sections. Yu Hsin and Jo wrote the discussion section collaboratively. Jo generated the plots in R while Yu Hsin edited and formatted the figures into panels and completed the figure captions. Yu Hsin and Jo collaboratively wrote the abstract and edited the manuscript.

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