Marmot gut microbiomes are stable against dietary variation

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Abstract
Animal gut microbiomes, particularly those of herbivorous mammals, are strongly shaped by the host diet. However, how dietary composition impacts gut microbiome variation across a population of wild hosts is unknown. To examine the relationship between gut microbiome composition and diet composition across individuals, we employed a multi-omic approach leveraging both 16S rRNA amplicon sequencing and plant DNA metabarcoding (tRNL primer) in 39 wild yellow-bellied marmot fecal samples from the Rocky Mountains. We utilized the 16 s rRNA primer to target microbes and the tRNL primer to target plants. Our results indicate that the marmot gut microbiomes appear to be stable against dietary variation, even across individuals with significantly different diets. We also show that colony membership significantly impacts marmot dietary variation, while age does not. Thus, while diet clearly plays a significant role in shaping mammalian gut microbiomes, our study suggests that diet composition within the same species has a minimal impact on gut microbiome variation, particularly in the absence of experimental manipulations and dietary interventions.

Introduction

Host-associated microbes, or "microbiomes", are largely ubiquitous across the animal kingdom (Colston 2017; MM Curtis 2011). Of particular interest is the gut microbiome, the most abundant and diverse community of host-associated microbes. Gut microbiomes are critical components of host physiology (Forsythe and Kunze 2013) through the regulation of metabolism (Nieuwdorp 2014), immune function (Round 2014; Shi et al. 2017), and animal behavior (Johnson 2020; Renson et al. 2020; Vernice et al. 2020). As such, gut microbiomes are important to understanding the survival and evolution of animal hosts.

The gut microbiome exhibits both plastic and resilient properties in the context of microbiome composition and diversity (McGuinness et al. 2022). Across a variety of host clades, gut microbiomes vary with host diet (Clayton et al. 2016; Dai et al. 2024; Klure and Dearing 2023; Mallott et al. 2018), environment (Degregori et al. 2021; Kim et al. 2021), or disease state (Nishida et al. 2018). Diet, in particular, has a significant impact on animal gut microbiome composition (Beam et al. 2021) that may lead to sustained changes in gut microbiome composition and diversity (Riaz Rajoka et al. 2017). In fact, host diet categories such as carnivore, herbivore, and omnivores can often predict gut microbiome composition across the animal kingdom (Claesson 2012; Leeming et al. 2019; Muegge 2011). In humans, dietary variation has led to direct changes in microbiota makeup associated with metabolic diseases (Bourdeau-Julien et al. 2023), and proliferations of specific bacteria have been documented in response to fructan uptake (Sonnenburg et al. 2010). Introduction of microbiota-accessible carbohydrates results in marked changes in the mouse gut microbiota (Smits et al. 2016), and diet explained up to 10% of microbial variation in humans (Salonen et al. 2014), highlighting how external host factors such as diet can shape gut microbiome composition. Thus, it is clear that diet plays a pivotal role in shaping the mammalian gut microbiome.

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However, gut microbiomes can also show remarkable stability, even in the face of dietary variation. For example, high/low fiber dietary interventions in human subjects led to temporary gut microbiome changes that reverted to previous compositions after only a few weeks (Wu 2011). Similarly, the initiation of the Nordic diet in humans who were then tested for the ratio of *Prevotella* to *Bacteroides* in the gut showed little effect on gut microbiome composition (Roager et al. 2014). Even fecal microbiome transplantations between the microbiota of zebrafish and mice led to the microbiomes returning to their original compositions, not that of the transplanted, indicating the importance of host phylogenetic stability over diet and environment (Rawls et al. 2006). In wild animals, factors such as host phylogeny and environment can significantly outweigh host diet in explaining gut microbiome composition, such as in mammals (Amato et al. 2019; Finnegan et al. 2024Nielsen et al. 2022), in birds (Baiz et al. 2023) and in fishes (Kim et al. 2021). Gut microbiome compositional variability associated with changes in host diet may be transient and short term (David et al. 2014), while the most abundant gut microbes, often termed the "core gut microbiome", remain unchanged during such interventions (Faith et al. 2013; Michel et al. 2023). However, high-resolution data to measure gut microbiome variability across individuals with varying diets is lacking, especially in non-human wild hosts. Understanding the extent to which gut microbiomes are stable against dietary variation in animal hosts is a timely endeavor for the field.

Gut microbiome studies examining non-human mammal gut microbiomes and diet often use broad diet categories which may lack the specificity necessary to define a causal link between diet and microbial makeup. For example, studies on mammalian gut microbiomes often only use three broad diet categories such as herbivore, omnivore, or carnivore rather than higher-resolution measures (Groussin et al. 2017; Ley et al. 2008; Muegge et al. 2011; Nishida et al. 2018). Moreover, many of these studies include multiple host species (Ley et al. 2008; Song et al. 2020; Youngblut et al. 2019), which, while important for comparative inference, can add phylogenetic variation that may confound with dietary variation in hosts. Additionally, existing studies investigating microbe–diet interactions have largely been limited to humans or animals in captivity which may provide different results compared to wild animal hosts (Bowerman et al. 2021). As such, we aimed to better isolate the effect of dietary variation in hosts by analyzing the covariation between gut microbiome and dietary composition among individuals within the same wild host species.

Wild hosts are of particular interest to this study, because they represent an approach to studying the naturally occurring diversity among gut microbes without the pressure of domestication or captivity. It is well established that living in the wild versus captivity not only impacts the health of species, but can establish changes to their microbiome (Malukiewicz et al. 2022). However, microbiome studies often use captive animals because of their ability to accurately document diet. Thus, we set out to investigate the relationship between the gut microbiome and diet diversity that exists in nature by studying wild hosts in the yellow-bellied marmots (*Marmota flaviventer*) and quantifying their diet through diet metabarcoding.

Diet composition has been traditionally quantified through observation or stomach content surveys, but these methods have limitations. More recently, dietary metabarcoding has proven an effective, less invasive approach to identify what animals eat. Importantly, because diet metabarcoding can be done on fecal samples, it is far less invasive than traditional stomach content analysis, which often requires euthanized hosts and can only detect prey that are visually identifiable (Barbato et al. 2019; Mychek-Londer et al. 2020).

To gain a better understanding of the host diet's role in shaping gut microbiome diversity, we employed a non-invasive multi-omic approach to investigate the association between DNA-derived diet composition collected with the trnL primer (plant identification) (Taberlet et al. 2007) and gut microbiome composition using the 16S rRNA primer (microbe identification) (Caporaso et al. 2011) in yellow-bellied marmots in the Rocky Mountains, an exceptionally well-studied social rodent (Armitage et al. 1976; Blumstein 2009).

Materials and methods

Microbiome sample processing and sequencing

Study species and sites

We studied yellow-bellied marmots in and around the Rocky Mountain Biological Laboratory (RMBL), located in the Upper East River Valley in Gothic,

Colorado, USA (38°77′N, 106°59′W). Marmots are hibernating mammals, and spend the summer months (May–September) feeding intensively to store up energy to survive the winter months (Blumstein 2009). As such, we sampled marmots during the summer months to accurately quantify their feeding behavior. All sampling procedures were conducted following Johnson et al[38]. Fecal samples were collected from trapped marmots and immediately bagged and put on ice to be stored at −20 °C within 2 h of collection and then transported on dry ice for 12 h to be stored at −80 °C for long term. To avoid contamination while handling, all fecal samples were collected from the pre-sterilized traps, while using sterile nitrile gloves and sterilized tweezers. Traps

were sterilized with bleach and ethanol in between sampling events. Over the course of the study, samples were collected from ten different colonies: five from higher elevation colonies (mean elevation 3043 m) and five from lower elevation colonies (mean elevation 2883 m), separated by a maximum distance of 4.9 km. However, for the purposes of this work, only a subset of these samples was taken for genomic analysis. From a larger subset of samples collected over five consecutive summers (2015–2019) in between the months of June and August, we chose a smaller subset, yielding 39 total paired samples, covering a range of metadata variables. In the field, the following metadata categories were collected: colony, valley position, animal ID, lactation status, mass, and age class. Because marmots are also social creatures and socialize within colonies (Armitage et al. 1976; Blumstein 2009), which can affect their gut microbiome composition (Pfau et al. 2023), we analyzed both colony and valley position as separate variables, where valley position served to capture environmental variation, whereas colony served to capture both environmental variation and social behavior.

Microbiome and diet metabarcoding sample processing and sequencing

We isolated DNA from fecal samples with Qiagen Power-Soil Extraction kits following the manufacturer's protocol (Germantown, Maryland, USA). Samples were subdivided into two distinct PCR amplification reactions prior to being sequenced separately. We generated 16S libraries using the 515F (GTGCCAGCMGCCGCGGTAA) and 806R (GGACTACHVHHHTWTCTAAT) primers targeting the V4 region of the 16S rRNA gene (Caporaso et al. 2011). To target plant DNA, we multiplexed these samples with primers targeting the P6 loop (5'-GGGCAATCCTGAGCCAA, reverse 5'-CCATTGAGTCTCTGCACCTATC) of the trnL chloroplast region—a universal plant marker widely used to identify a broad range of plant taxa (Taberlet et al. 2007), including digested plants for diet metabarcoding purposes (Kartzinel et al. 2015). Samples underwent PCR, in triplicate 25 l reactions, using a Qiagen Multiplex PCR kit with the following thermocycler conditions for 16S primers: 1 cycle of 94 °C for 3 min; 35 cycles of 94 °C for 45 s, 50 °C for 60 s, and 72 °C for 90 s; and 1 cycle of 72 °C for 10 min (Thompson et al. 2017). Samples exposed to trnL primers underwent identical laboratory methods except for the first PCR where trnL PCR samples underwent the following reaction parameters: 1 cycle of 95 °C for 10 min; 35 cycles of 95 °C for 30 s, 50 °C for 30 s, and 72 °C for 2 min. Triplicate reactions were pooled after confirming amplification success through gel electrophoresis, dual-indexed samples using the Nextera UD Index Kit (Ilumina, San Diego, USA), and then purified with OMEGA Bio-Tek MagBind magnetic beads (Norcross, Georgia, USA). Laragen (Culver City, California, USA) performed quantification and pooling to create libraries with equimolar sample concentrations to ensure that both the trnL library and 16S library received equal coverage. Multiplexed libraries were paired end sequenced (300 bp per sequence) on an Illumina Miseq v3 at Laragen. We used negative controls from the DNA extraction and PCR processes throughout sample processing and added these to the final pooled library for sequencing.

Bioinformatic processing

Plant DNA processing

From 39 original paired samples from fecal samples, we obtained 1,603,048 which were then pared down after initial pruning of the datasets for high-quality reads. First, we aligned forward and reverse reads into a single file, which we completed using the MICCA (MICrobial Community Analysis) platform (Albanese et al. 2015). After merging, we removed any remaining PCR primers from the sequences by filtering for the known sequences outlined in the sample processing section. Next, using MICCA, we filtered sequences with a max ee rate (i.e., the quality of read in terms of confidence) of 0.75 and a minimum length of 40, filtering for % of reads. Using MICCA, we created operational taxonomic units (OTUs) with 97% sequence similarities. Rarefaction was applied to the samples such that each sample was appropriately downsampled to the 1000 read sequencing depth available using the vegan package (v2.5–7) and removing any samples falling below this minimum. This selected depth allowed for retention of 100% of our samples while maintaining the diversity among captured features (Fig S2A,B). We assigned taxonomy using a naive Bayes taxonomy classifier trained on the CaleDNA database for TRNL primers (Meyer et al. 2021). We then performed multiple sequence alignment to determine the phylogenetic assembly of the OTUs derived from the taxonomical assignment using MAFFT (Katoh et al. 2002).

Microbial DNA processing

Using qiime2 (Boylen et al. 2019) instead of MICCA, sequences were first aligned forward and reverse, demultiplexed by sample barcodes before adapters/primers were removed using qiime cutadapt and data denoised using dada2 (Callahan et al. 2016) and merged into a feature table for ASV analysis. Filters were applied to remove low abundance features, removing features with a total abundance of less than ten summed across all samples. Following these initial pruning steps and filtering out of low-quality reads, data was rarefied to the 1000 read sample depth within microbial samples for analysis using the qiime2 feature table rarefy function, removing any samples below this threshold.

Using the SILVA database Bayes taxonomy classifier, we performed a taxonomic assignment. Qiime2 was utilized to remove reads with non-bacterial assignment following these feature table productions.

Sequencing and filtering results

Diet data resulted in a combined total of 203,149 forward and reverse reads across the 39 collected feces samples, averaging 5209 reads per sample. Our negative sample generated less than ten reads. From these initial reads, filtering for quality and removal of primer sequences left 163,048 reads for further analysis, averaging 4181 reads per sample. Rarefaction performance left 24% of the OTU table, which began with a range of 832–31,281 reads per sample. A total of 6.41% of reads were assigned as unclassified at the family level, while 58.2% of reads were labeled as unclassified at the species level.

For microbial sequencing, initial reads yielded a total of 6,371,587 forward and reverse paired reads with a mean sequence read depth of 41,405 reads per sample. After filtering for high-quality reads, 1,614,799 forward and reverse reads remained. Rarefaction was used, leading to the filtering out of 76.4% of reads from the ASV table, which began with a range of 277–31,497 reads per sample. Following taxonomic assignment, a total of 9.7% of reads were assigned as unclassified at the family level, with 95% of reads being labeled as unclassified at the species level.

Statistical analyses

Statistical analysis was conducted in R using the phyloseq package (McMurdie and Holmes 2013).To determine the relative abundance of each taxonomic group in both diet and microbial samples, we generated relative sum abundance plots on the phylum and family levels. To produce a sense of within-sample diversity, we utilized the estimate_richness () function of the phyloseq (v1.30.0) package to generate alpha diversity plots from each samples' rarefied OTU (diet) or ASV (microbe) tables.

To assess the beta diversity across diet samples, we constructed distance matrices using unweighted Uni-Frac (Lozupone et al. 2011) and Bray–Curtis (Beals et al. 1984). Then we visualized these matrices through a principal coordinate analysis (PCoA). We focused on the UniFrac metric of beta diversity, since this metric captures microbial diversity at multiple taxonomic scales, and the host diet acts on various microbial taxonomic scales (Groussin et al. 2017). We produced the distance matrices with the packages Phyloseq (v1.30.0) and vegan (v2.5–7) using the statistical software R (v4.2.2) and then repeated these steps separately to generate PCoA plots for microbial data. To quantify and analyze beta diversity,

we employed a PERMANOVA analysis for a diet and gut microbiome comparison using the same packages with the adonis2 function employed. Each factor was analyzed within its own PERMANOVA model since a multi-factor PERMANOVA would involve too few samples per treatment. We selected metadata factors of particular interest (age, colony, colony position) and computed ANOVA-like differences from a two-way analysis using the ALDEx2 package (Fernandes et al. 2013). To better interpret PERMANOVA results, we employed a beta dispersion test for microbe and diet using the betadisper function in the vegan package to test for the assumption of homoscedasticity. To account for limitations in sequencing and to control spurious correlations, we transformed the OTU tables using the centered log ratio, or CLR (Aitchison 1982; Gloor et al. 2017).

We note two mechanisms for examining the environment as a metadata variable, the colony itself and its relative geographic position to the valley of sample collection. While colonies may provide a higher-resolution grouping for analyzing differences in microbial and diet composition as well as capturing social behavior, the resulting small sample sizes make downstream analysis weaker in effect. Thus, we supplement this approach by also considering "valley position" as a factor, with colonies being grouped as either up-valley or down-valley colonies. As described earlier, these two valley locations are distinct from one another and differ significantly in elevation.

To analyze the covariation between host diet and gut microbiome composition, we conducted a multiple regression on matrices (MRM) (Breiman 2001) analysis using the MRM function from the ecodist package (Goslee and Urban, 2007). MRM was conducted using a variety of distance matrices for microbiome compositions (UniFrac, Jaccard, and Bray–Curtis were tested) and a host diet distance matrix (using the same distance computations) to confirm the results using multiple methods of calculating distances.

Results

Yellow-bellied marmot diet composition

At the phyla level, Streptophyta dominated the diet composition of the marmot fecal samples, encompassing nearly all samples with approximately 92% of the recorded phyla. An additional 8% were labeled as unclassified. At the order level, Fabales made up around 24%, Asterales 23%, Ranunculales 15.46% of samples, Rosales 6.7%, Apiales 6.3%, and Malpighiales 5.5%. At the family level, marmot diets comprised Fabaceae (peas) at 27%, Asteraceae (sunflowers) at 23%, Rosaceae (roses) at 6.8%, and Apiaceae (celery

Fig. 1 A Relative family abundances comprising the yellow-bellied marmot diet. Raw sequence counts were transformed to relative abundances for this plot and taxonomic assignments were conglomerated to the family level. **B** Images of the common plant families found in yellow-bellied marmot diets. a) Fabaceae, b) Asteraceae, c) Rosaceae, d) Apiaceae

(a) Relative Family Abundance Diet

(b)
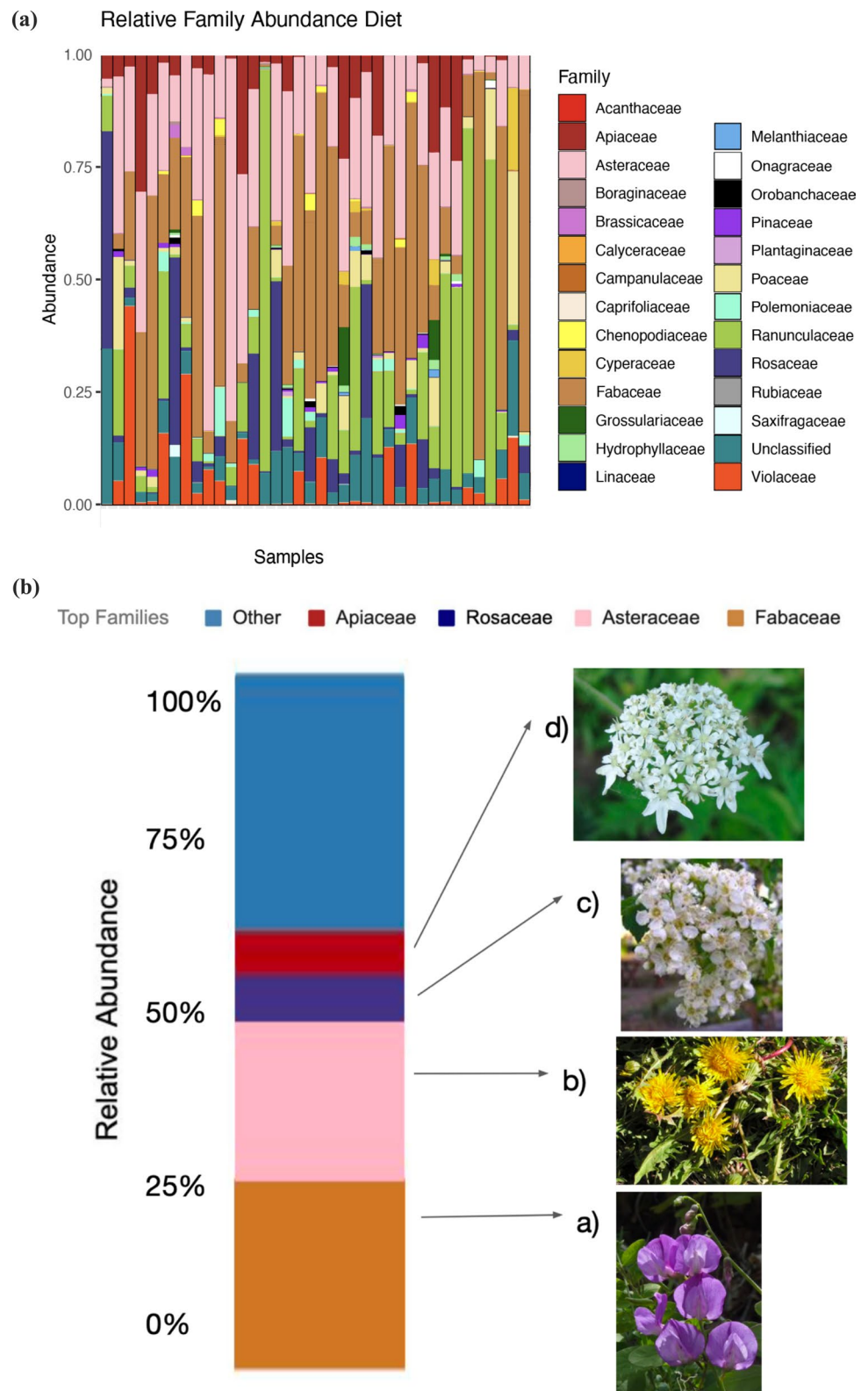
or carrots) at 6.2% (Fig. 1A, B). Marmot diet compositions did not significantly differ in alpha diversity across habitat (Fig S1A).

Yellow-bellied marmot gut microbiome composition

For microbial sequencing, the phyla level was dominated by Firmicutes (53%), Bacteroidetes (28%), and Tenericutes (13%) (Fig. 2A). At the order level, the major groups were Clostridiales at 64%, Bacteroidales at 26%, and Bacilli at 9%. Finally, at the family level, Ruminococcaceae was around 21%, S247 around 14%, unclassified at around 10%,

and Bacteroidaceae at 6% (Fig. 2B). Marmot gut microbiome compositions did not significantly differ in alpha diversity across habitat (Fig S1B).

Marmot gut microbiome composition does not covary with diet composition

We used MRM (multiple regressions on matrices) to determine the correlation between the composition of the diet of a sample and the corresponding microbial community. Using several distance matrix methods, we found no significant covariation between marmot gut microbiome and

Fig. 2 **a** Relative abundance of gut microbial phyla in marmot fecal samples. **b** Relative abundance of gut microbe families in marmot fecal samples
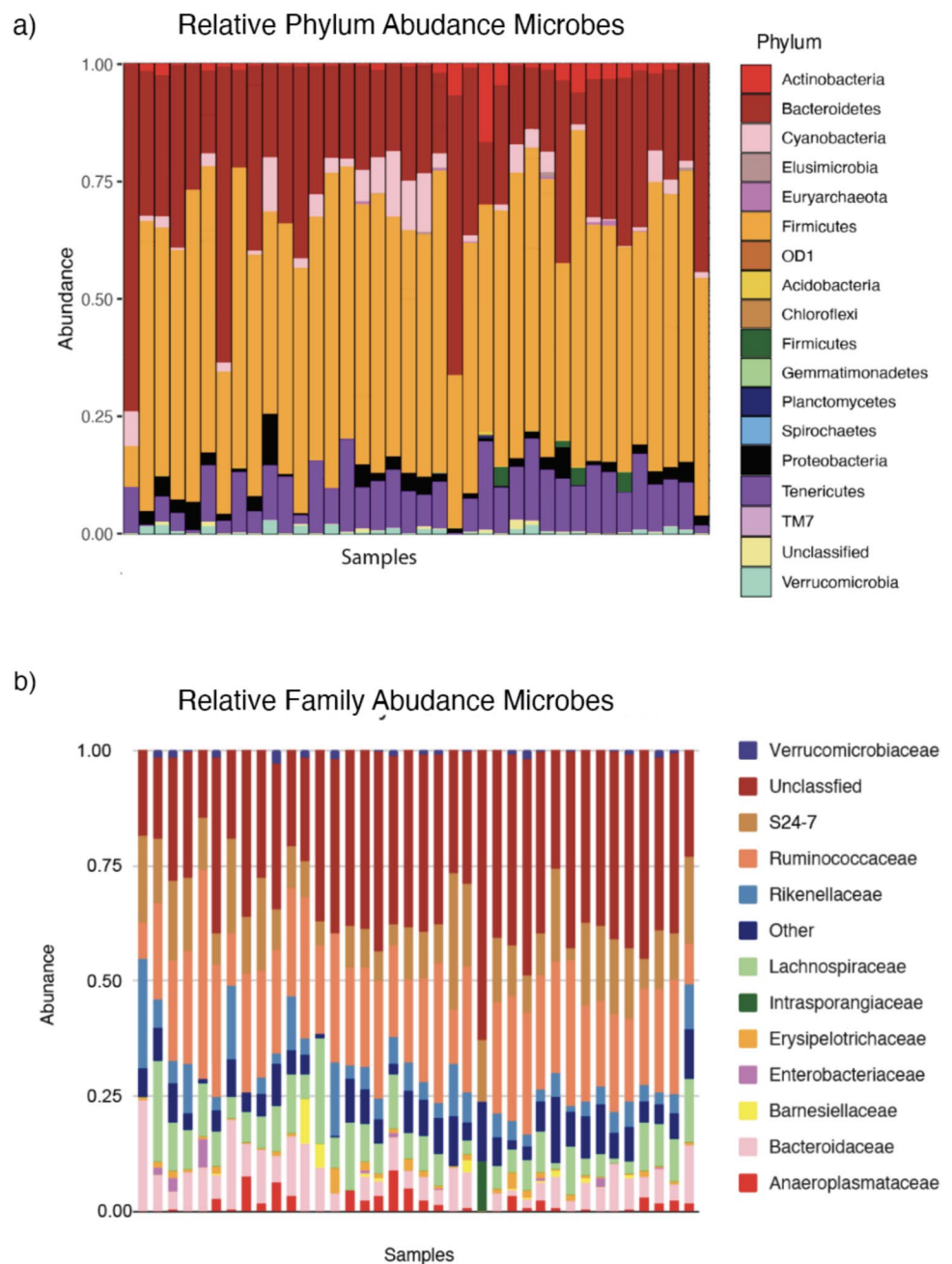
Table 1 MRM results between all distance matrix combinations

Distance matrix type—diet	Distance matrix type—microbes	P value
Unweighted UniFrac	Unweighted UniFrac	0.5
Bray–Curtis	Bray–Curtis	0.9
Jaccard	Jaccard	0.1
Bray–Curtis	Unweighted UniFrac	0.1
Jaccard	Unweighted UniFrac	0.7
Bray–Curtis	Jaccard	1
Jaccard	Bray–Curtis	0.7

diet compositions (Table 1). These results indicate that variation in gut microbiome is not driven by diet in our samples.

Colony and valley position impacts dietary variation

We examined each of the existing metadata factors of age, date of sample collection, year of sample collection, colony, valley position, lactation status, and mass to determine if marmot diet beta diversity correlated with any of them. Of these factors, colony and relative position within valley (up valley or down valley) had the largest impact on dietary beta diversity ($N = 39$, $R^2_{\mathrm{PERMANOVA}} = 0.32157$, $P_F = 0.001$ (Table 2) and $N = 39$, $R^2_{\mathrm{PERMANOVA}} = 0.14104$, $P_F = 0.001$, (Table 2), respectively). A PERMANOVA by age also revealed a weaker, but significant correlation with marmot dietary beta diversity ($N = 39$, $R^2_{\mathrm{PERMANOVA}} = 0.06245$, $P_F = 0.015$, Table 2). Visually, samples from the "Gothic Town" colony clustered together the most, relative to the more dispersed nature of the rest of the marmot samples in the PCoA space for diet (Fig. 3).

Microbiome composition similar across individuals

To explore drivers of microbial variation, we ran another set of PERMANOVA analyses examining the same metadata factors but for the microbial composition of the samples. Unlike diet, there was a minimal impact on microbiome diversity by colony ($N = 39$, $R^2_{\mathrm{PERMANOVA}} = 0.16583$, $P_F = 0.06$, Table 2) and the valley position (which can

be viewed as a more broad categorization of the colony space) showed no explanatory power ($N = 39$, $R^2_{\mathrm{PERMANOVA}} = 0.02889$, $P_F = 0.338$, Table 2). Similarly, PCoA results did not reveal any significant differences in microbe composition for any of the metadata factors that we examined during sample collection. Repeating the analysis with different distance calculations (Bray–Curtis and Jaccard) revealed the same pattern of colony/valley position driving differences in diet, but only weakly, or not at all, explaining differences in microbiome composition (Table S1 and S2).

Beta dispersion results

To assess the degree of group dispersions among diet and microbe samples, we ran a beta dispersion analysis on diet and microbiome samples across both colony and valley position (up- or down-valley). Testing these groups for dispersion in both microbial composition and diet using unweighted UniFrac distance matrices (Table 3), only colony was significant in testing microbiome homogeneity of variance across microbiome samples alone. We also achieved similar results with a weighted UniFrac distance matrix (Table S3). Repeating this analysis with different distance calculations (Bray–Curtis and Jaccard) corroborated that between metadata, group differences did not drive variance differences in diet composition (Table S1 and Table S2). However, Bray–Curtis and Jaccard distance measurements find some significant results showing colony and valley position to drive variance difference between metadata groups (Table S1 and Table S2). When the marmot meadow colony, a low sample size colony ($n = 3$), is excluded from analysis, these results are no longer significant (Table S4).

ALDEx2 results

To further explore beta-diversity differences, we examined whether specific OTUs are unique to individual locations or by age demographics. For diet composition, one OTU (Plantae, Streptophyta, unclassified, Pinales, Pinaceae, Abies, unclassified) was only found in yearling marmots (Fig. 4A) and was also only found in the "Gothictown"

Table 2 PERMANOVA test results for valley position, colony and age class across gut microbiome and diet samples. (UniFrac unweighted distance matrix)

| Distance matrix	Metadata	R squared	Df	F	Sum of squares	Pr(> F)	Sig code
Diet UniFrac	Colony	0.32157	5	3.0336	1.5712	0.001	**
Diet UniFrac	Age class	0.06245	1	2.3979	0.3051	0.015	*
Diet UniFrac	Valley position	0.14104	1	5.9111	0.6891	0.001	***
Microbe UniFrac	Colony	0.16583	5	1.2723	1.1598	0.06	
Microbe UniFrac	Age class	0.044	1	1.6568	0.3077	0.052	
Microbe UniFrac	Valley position	0.02889	1	1.0709	0.202	0.338	
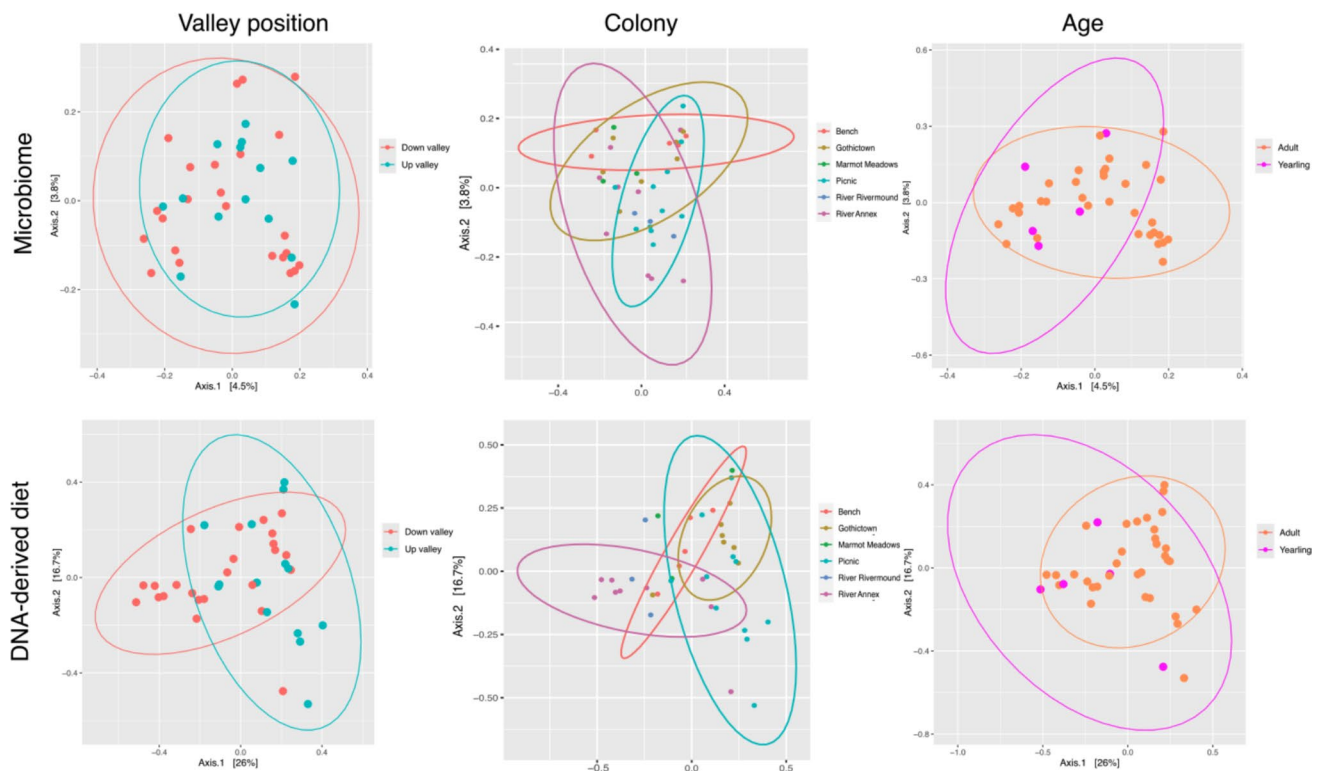
Fig. 3 Principal coordinate analysis of microbiome (top) diet samples (bottom) grouped by the relative position in the valley of collection, the area in the field they were collected (colony), and the age of the marmot. Plot generated using a Bray distance matrix for diet and microbiome. Lassos represent 95% confidence intervals

Table 3 Beta dispersion results for microbes and plants across colony position using permutation test for homogeneity of multivariate dispersions. (UniFrac unweighted distance metric)

Distance matrix	Meta data	Mean squared	Df	F	Sum of squares	Pr(> F)	Sig code
Diet UniFrac	Colony	0.0075166	5	1.028	0.037583	0.406	
Diet UniFrac	Valley position	0.0000184	1	0.0025	0.000018	0.96	
Microbe UniFrac	Colony	0.0075302	5	2.889	0.037651	0.035	*
Microbe UniFrac	Valley position	0.0014396	1	0.3219	0.00144	0.565	

colony (Fig. 4B). Similarly, for microbes, Bacteria, Bacteroidetes, Bacteroidia, Bacteroidales, S24-7, unclassified, unclassified and Bacteria, Bacteroidetes, Bacteroidia, Bacteroidales, Bacteriodaceae, Bacteroides, unclassified were only observed in yearling marmots (Fig.4 C), but not differentially expressed in colonies.

Discussion

Diet is a major determinant of gut microbiome composition in animal hosts (2011);Ley et al. 2008; Muegge et al. 2011 however, our work demonstrates that the gut microbiome of wild hosts may be more resistant to changes in diet than previously thought. We generate a high-resolution multi-omic dataset of yellow-bellied marmots in the Rocky Mountains from fecal samples to extract both diet and microbiome compositions utilizing the tRNL and 16 s rRNA loci, respectively. The results from our sequencing are consistent with previous work on the yellow-bellied marmots whose main diet comprises Ranunculaceae and Asteraceae at the family level (Armitage 2003, Frase and Armitage 1989) and a microbiome consisting largely of Bacteroidetes and Firmicutes (Degregori et al. 2021). The diversity of the sequences in our microbial results is largely in agreement with other studies which have found the microbial diversity of an organism to be unaffected by diet (Chen et al. 2021; Rinninella et al. 2019).

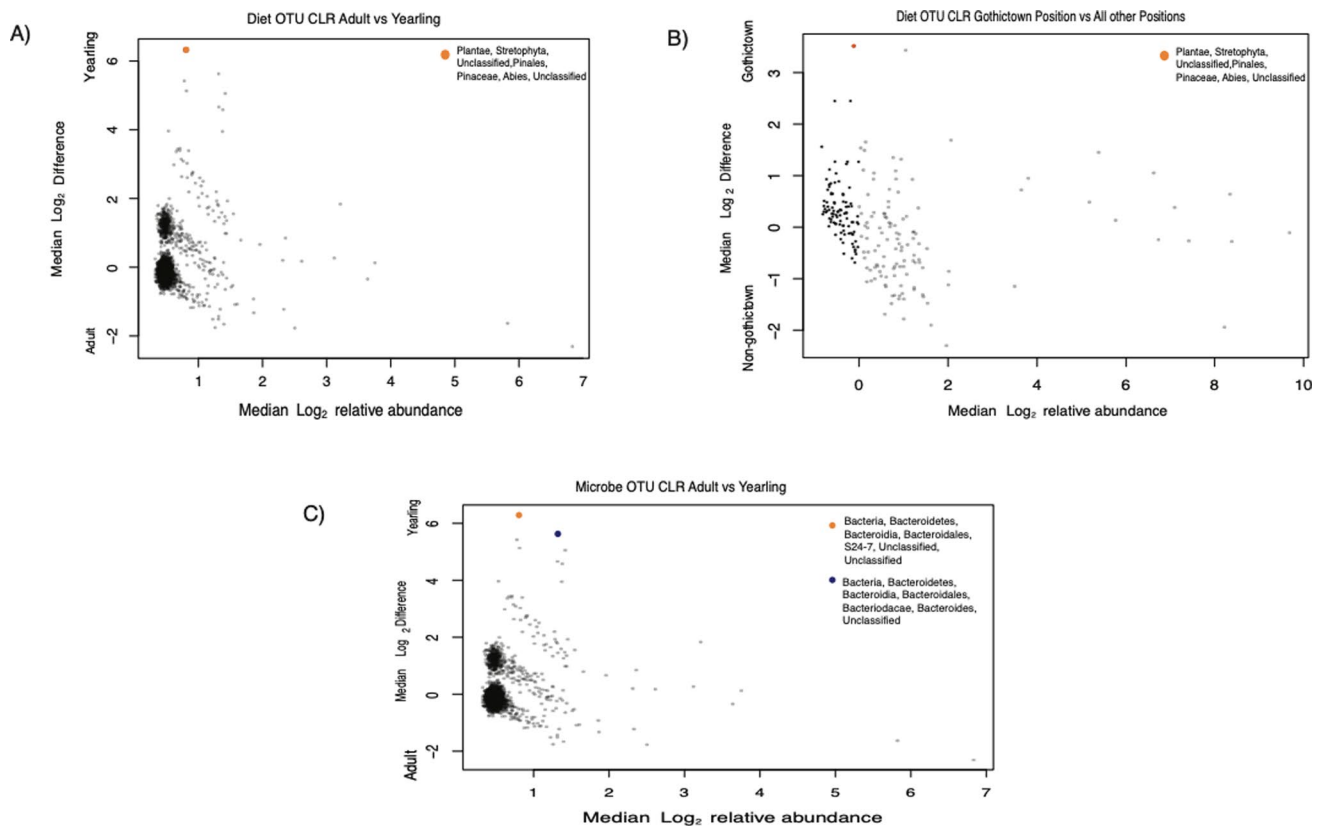Despite significant dietary variation across marmots from different colonies, we find that marmot gut microbiome

Fig. 4 **A** Diet operational taxonomic units (OTUs) expression in marmot samples based on age. Significant points (i.e., OTU's differential expressed in one group versus the other) are highlighted in purple and given taxonomy (Plantae, Stretophyta, Unclassified, Pinales, Pinaceae, Abies, Unclassified). **B** Diet operational taxonomic units (OTUs) expression in marmot samples based on location of sample being inside of the Gothic town site or anywhere else. The only significant points (OTUs with significantly higher or lower proportions) are highlighted in orange and given taxonomy. (Plantae, Stretophyta, Unclassified, Pinales, Pinaceae, Abies, Unclassified). **C** Microbe operational taxonomic units (OTUs) expression in marmot samples based on age (yearling or adult). Significant points (OTUs with significantly higher or lower proportions) are highlighted in blue and orange. Blue—Bacteria, Bacteroidetes, Bacteroidia, Bacteroidales, S24-7, unclassified, unclassified and orange—Bacteria, Bacteroidetes, Bacteroidia, Bacteroidales, Bacteriodaceae, Bacteroides, unclassified

variation is unchanged across individuals. Thus, host traits beyond feeding strategy have a greater effect than host diet on marmot gut microbiome composition. Across two methods of assessing the colony location, we find no significant differences in microbiome samples by metadata factors of interest. This result provides some evidence that contrasts previous work demonstrating the relative importance of the social environment in shaping the marmot gut microbiome (Pfau et al. 20232023); however, this could also reflect the smaller sample size of the study.

Our work also provides an important contrast to other findings that show dietary variation significantly shapes gut microbiome diversity, potentially due to our sampling a single host or species rather than a range of host taxa. Kartzinel et al., for example, found that diet composition was significantly correlated with microbiome composition across 33 host species of herbivorous mammals (Kartzinel et al. 2019). Sampling a diversity of hosts may capture enough dietary diversity to find a significant effect in

shaping gut microbiome composition. Our results imply that the composition of a single organism's diet of the same species does not impact gut microbiome composition in the same way and suggests some degree of hysteresis, or lag between diet and microbial community composition. In humans, (David et al. 2014) demonstrated that even short-term changes to the diet can make significant changes to the composition of the gut microbiome. Thus, it is possible that marmot gut microbiomes may shift due to diet, but may require extreme dietary shifts to result in such a prolonged effect. The gut microbiome may also reflect the average diet of a marmot over an entire feeding season, in contrast to the specific diet we quantified using metabarcoding at a given moment of sample collection. To help compensate for this disparity between the two data types, we ensured that our fecal samples spanned the entire feeding season (May–August) and included a range of habitat types and ages. The fact that marmot gut microbiomes did not correlate with age, dietary variation, or

habitat implies that marmot gut microbiomes are stable throughout the feeding season.

Given our current data, the homogenous nature of marmot gut microbiome composition may be better explained by features such as host environment or ontogeny. Studies finding significant effects of host diet shaping gut microbiome composition often do so when comparing hosts across many families or even classes—and even so, still find strong effects of host environment and phylogeny in addition to diet (Baiz et al. 2023; Groussin et al. 2017; Ley et al. 2008; Youngblut et al. 2019). Within humans, there are examples of gut microbiome stability across individuals (2011);Leeming et al. 2019; Lozupone et al. 2011 however, our study provides novel evidence that gut microbiomes are stable against dietary composition across individuals of a wild non-human species consuming their natural diet.

Our work falls into the scope of existing literature surrounding wild hosts, which has been challenging to analyze given the wide range of confounding variables such as social behavior, seasonal changes, and habitat degradation (Zhu 2022; Hicks et al. 2018). A large number of studies examining the interaction between the gut microbiome and an organism's diet have been restricted to humans (Leeming et al. 2019; Mansour et al. 2021; Valdes et al. 2018) or domesticated organisms, such as mice (Reese et al. 2021; Wang et al. 2021). Our study provides an example of an understudied wild host unaffected by controlled dietary interventions or a laboratory environment. As a result, our study shows that without extreme interventions in controlled laboratory settings, diet may not play as large a role in shaping individual gut microbiome variation in small mammals, such as marmots.

However, the multi-omic approach to this study is not without limitations, and we recognize the importance of selecting a primer for isolating the diet and microbiome composition. We focused on a subsection of diet, i.e., herbivory, since the trnL primer only targets plant DNA. Previous studies have shown that the herbivorous gut microbiome is largely adapted to metabolize carbohydrate substrates (Flint et al. 2015; Newsome et al. 2020). Regarding the diet of marmots, it is possible that while different plant species may be from distinct phylogenies, they may share similar carbohydrate structures. Thus, the marmot gut microbiomes in our study may simply be encountering biochemically similar plant species that can undergo the same fermentative processes in the gut. We also recognize the difficulty in assessing diet composition for a single time point, which is an inherent simplification of the diet of marmots. Future studies should employ shotgun sequencing to better ascertain gut microbiome function of yellow-bellied marmots to better understand how the gut microbiome aids in marmot digestion.

Declarations

Conflict of interest The authors declare no conflicts of interest.

Ethical approval Marmots were studied under IACUC protocols approved by UCLA (2001-191) and were trapped under permits approved by the Colorado Department of Parks and Wildlife (TR917).

Consent to participate N/A.

Consent for publication N/A.

Student author nomination The question as to how much diet impacts gut microbiome composition has been hotly debated since the beginning of the gut microbiome field. Our work suggests that across individuals, the gut microbiome is quite stable and does not correlate with dietary composition even when those individuals consume different diets. Cecilia McCormick, the first-author, was able to execute this project while also utilizing a novel bioinformatic pipeline to analyze microbial and plant DNA simultaneously. As an undergraduate who is only in her third year, this is an incredible feat, and we proudly nominate her for the Oecologia Highlighted Student Paper.

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