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Research Article

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Firmicutes and Bacteroidetes contribute to mass gain variation in female obligate hibernators

Samuel Degregori[1], Gina C. Johnson[1], Paul H. Barber[1], Daniel T. Blumstein[1,2,*]

[1]Department of Ecology and Evolutionary Biology, University of California, 621 Young Drive South, Los Angeles, CA 90095-1606, United States
[2]The Rocky Mountain Biological Laboratory, Box 519, Crested Butte, CO 81224, United States

*Corresponding author: Department of Ecology and Evolutionary Biology, University of California, 621 Young Drive South, Los Angeles, CA 90095-1606, United States. Email: marmots@ucla.edu
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Abstract

Obtaining body condition is an important life history challenge that directly impacts individual fitness and is particularly important for hibernating animals, whose maintenance of adequate body fat and mass is essential for survival. It is well-documented that host-associated microorganisms play a vital role in animal physiology and behavior. Recent work demonstrates that gut microbes are associated with fat accumulation and obesity, particularly the phyla Firmicutes and Bacteroidetes. The focus of most microbiome studies has been on human health or involved lab-reared animals used as a model system. However, these microbes likely are important for individual fitness in wild populations and provide potential mechanistic insights into the adaptability and survival of wildlife. Here we tested whether symbiotic microorganisms within the phyla of Firmicutes and Bacteroidetes were associated with summer mass gain in an exceptionally well-studied wild population of yellow-bellied marmots (*Marmota flaviventer*) by analyzing 207 fecal samples collected over 5 summer active seasons. Results showed that marmots with higher mass gain rates had a greater relative abundance of Firmicutes. In contrast, a higher relative abundance of Bacteroidetes was associated with lower mass gain rates, but only for marmots living in harsher environments. Similar patterns were found at the family level where Ruminococcaceae, a member of Firmicutes, was associated with higher mass gain rates, and Muribaculaceae, a member of Bacteroidetes, was associated with lower mass gain rates in harsher environments. Although correlative, these results highlight the potential importance of symbiotic gut microbiota to mass gain in the wild—a trait associated with survival and fitness in many taxonomic groups.

Key words: body condition, fitness, hibernation, life history, microbiome.

The maintenance of sufficient body condition is a major life history challenge shared by animals with important implications for individual fitness (Gaillard et al. 2000; Green 2001; Schulte-Hostedde et al. 2001). Animals in good condition can endure longer fasting periods (Atkinson and Ramsay 1995), are more likely to survive long migrations (Merilä and Svensson 1997), maintain a more responsive immune system (Navarro et al. 2003), have increased fecundity (Tammaru et al. 1996), and enjoy higher mating success (Cotton et al. 2006). Body mass, in particular, can have a large effect on individual survival in many taxa (Jakob et al. 1996; Schulte-Hostedde et al. 2011). For instance, larger body mass increases the probability of survival in bighorn sheep (*Ovis canadensis*; Festa-Bianchet et al. 2011), canvasbacks (*Aythya valisineria*) (Haramis et al. 1986), and great tits (*Parus major*; Gosler et al. 1995).

While a variety of factors including food availability, predation risk, and temperature influence individual body mass (Lima 1986), a growing body of literature suggests that host-associated microorganisms—collectively referred to as "microbiomes"—also play a key role in shaping host physiology (Neish 2009; Kinross et al. 2011; Hird 2017). The complex network of microbes that reside in the vertebrate gastrointestinal tract influences metabolic activity of the host and affects numerous aspects of physiology, anatomy, and behavior (Cryan and Dinan 2012; Nicholson et al. 2012). Research on humans and other animals suggests a strong link between the intestinal microbiome and mass gain (Ley et al. 2006; Tsai and Coyle 2009; Million et al. 2012), with shifts in the dominant phyla of gut bacteria associated with obesity (Ley et al. 2005, 2006; Turnbaugh et al. 2006, 2008; Ley 2010).

Most gut bacteria in vertebrate hosts belong to 4 major phyla; Firmicutes, Bacteroidetes, Proteobacteria, and Actinobacteria (Tilg and Kaser 2011)—sometimes referred to as the "core microbiome" (Turnbaugh et al. 2009; Hird et al. 2015). Studies in mammals suggest that shifts in relative abundance, with more Firmicutes and fewer Bacteroidetes, are associated with fat accumulation and potential for obesity—in contrast, weight loss and leanness are associated with higher relative abundance of Bacteroidetes (Ley et al. 2005; Turnbaugh and Gordon 2009). That relative abundance of

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Firmicutes and Bacteroidetes influences obesity suggests that gut microbiomes can affect energy extraction from the diet (Ellekilde et al. 2014), with strong implications for individual fitness, especially for animals whose survival depends on developing and maintaining adequate fat stores. Furthermore, the association of Firmicutes and Bacteroidetes with obesity has been brought into question in recent years (Indiani et al. 2018; Fabien Magne et al. 2020), calling for further well-designed studies.

Developing fat stores is critical to hibernating animals, whose long-term survival and growth depend on adequate body fat accumulation (Turbill et al. 2011). Hibernation involves dramatic seasonal changes in individual food consumption, body mass, and energy expenditure (Lyman and Chatfield 1955; Florant et al. 2004). Moreover, hibernation coincides with a shift in gut microbial communities across a diversity of taxa—including mammals (Sonoyama et al. 2009; Dill-McFarland et al. 2014; Malinčiová et al. 2017), amphibians (Kohl and Yahn 2016; Weng et al. 2016), and reptiles (Tang et al. 2019)—suggesting that gut microbiota may have functional importance in hibernating animals (Carey and Assadi-Porter 2017).

Yellow-bellied marmots (*Marmota flaviventer*) are obligate hibernators that must accumulate sufficient fat stores to survive a 6- to 8-month period of hibernation (Armitage 1998; Armitage et al. 2003). Marmots lose up to half of their body mass during hibernation (Armitage et al. 1976); thus, mass gain during the active season is essential for survival. Moreover, adequate fat stores are essential for reproductive female marmots to give birth, directly influencing individual reproductive fitness (Andersen et al. 1976). Environmental conditions largely explain variation in mass gain in marmots (Maldonado-Chaparro et al. 2015), but age, sex, diet, food availability, and body size can also play a role (Armitage et al. 1976, 2003). However, given seasonal shifts in gut microbiomes of other hibernating animals (Stevenson et al. 2014a, 2014b; Sommer et al. 2016), and that microbiome composition can influence mass gain (Ley et al. 2005; Turnbaugh and Gordon 2009; Ellekilde et al. 2014), it is possible that gut microbiome composition could influence the survival and reproductive fitness of individual marmots.

We examined the association of gut bacteria and mass gain rate in an exceptionally well-studied wild population of yellow-bellied marmots. This population has been continuously monitored since 1962, providing long-term data on mass gain during the active season, overwinter and summer survival, and reproductive success. Juveniles emerge from their natal burrow in late June to early July and must rapidly gain mass and body size to survive their first hibernation, despite not reaching full body size until their second year. Yearlings tend to show the greatest change in mass as they gain fat to survive hibernation but also undergo somatic growth to reach adult body size during their second summer active season. Adults (defined as reproductively mature females in their third year of life or older) have typically reached full body size, so they only need to accumulate sufficient fat stores during their summer active season (Armitage et al. 1976). Thus, age—in addition to factors such as chronic stress and spatiotemporal variation—plays a critical role in mass gain (Armitage 2014) and individual survival (Ozgul et al. 2006; Wey et al. 2015).

Using 16S rRNA microbial metabarcoding, we examined microbial composition in free-living marmots to estimate the relative effects of microbiome composition and environmental factors in explaining variation in mass gain rates. Specifically, we tested the hypothesis that higher Firmicutes relative abundance would be associated with greater mass gain while higher Bacteroidetes relative abundance would be associated with lower mass gain rates. Given the importance of individual host biology and ecology and their effects on body condition, we also tested whether age, colony, and habitat elevation interacted with the marmot gut microbiome to influence mass gain rate.

Materials and methods

Study species and site.

We studied yellow-bellied marmots in and around the Rocky Mountain Biological Laboratory (RMBL), located in the Upper East River Valley in Gothic, Colorado, United States (38°77′N, 106°59′W). Marmots were trapped by placing Tomahawk live traps near burrow entrances. After capture, the marmots were transferred to cloth handling bags to measure their body mass (to the nearest 10 g), and to determine their sex and reproductive status (Blumstein et al. 2006). Because marmot masses in this study ranged from 295 to 4,463 g (mean = 2,337 g), the 10 g margin of error still allowed for an accurate quantification of marmot weights in this data set. Each marmot was given a set of unique ear-tag numbers and their dorsal pelage marked with Nyanzol fur dye for identification from afar. Fecal samples are easily collected throughout the season when animals are live-trapped. When feces were found in traps, they were routinely collected in a plastic bag, immediately put on ice, and subsequently frozen at –20 °C within 2 h of collection. To ensure freshness and minimize the effects of decomposition, we only collected recently deposited feces (which also included those that were excreted while we were handling the marmots). Samples were then transported from the field on dry ice and stored at –80 °C in the lab for long-term preservation. All field protocols were approved by the UCLA IACUC (2001-191); live-trapping was approved by the Colorado Department of Parks and Wildlife (TR917).

To capture variation within the active season, we also selected paired female samples from a large archive of previously collected fecal samples although for some individuals only a single sample was available. We focused on females, because both overwinter survival and reproduction the next year depend on body condition (Andersen et al. 1976). To account for social interactions between individuals—which can influence gut microbiome variation (Moeller et al. 2016), and habitat differences including elevation, we collected samples from 10 different colonies: 5 higher-elevation colonies (mean elevation 3,043 m) and 5 lower-elevation colonies (mean elevation 2,883 m), separated by a maximum horizontal distance of 4.9 km. Although there is only an average difference of 160 m in altitude between these sites, the phenology of these locations differs substantially, resulting in emergence from hibernation and mating approximately 2 weeks earlier in lower-elevation colonies (Blumstein 2009).

We selected samples collected closest to 1 June and 15 August, dates that fall within the period of linear mass gain during their active season (Heissenberger et al. 2020), although some samples were collected as early as May and as late as September. To maximize statistical power and test for consistency across time, we selected samples across a 5-year span (2015 to 2019), yielding 207 total samples representing 71 individuals. Because each age class faces unique ecological challenges (Armitage et al. 1976; Heissenberger et al. 2020), we sampled from multiple age groups, totaling 25 juvenile, 67 yearling, and 109 adult samples (Table 1).

Table 1. Characteristics of yellow-bellied marmots among selected samples (n = 201).

Characteristics	Number of samples (unique individuals)				
Year	2015	2016	2017	2018	2019
Age class					
Adult	11 (6)	13 (8)	26 (13)	22 (11)	37 (20)
Yearling	4 (3)	20 (11)	8 (4)	9 (5)	26 (14)
Juvenile	2 (1)	3 (2)	7 (4)	0	13 (7)
Valley position					
Up-valley	16 (9)	29 (16)	30 (15)	20 (10)	27 (14)
Down-valley	1 (1)	7 (5)	11 (6)	11 (6)	49 (27)

Microbiome sample processing and sequencing

We isolated bacterial DNA from fecal samples with Qiagen Powersoil Extraction kits following the manufacturer's protocol (Germantown, Maryland). We generated 16S rRNA libraries using the 515F (5′-GTGCCAGCMGCCGCGGTAA) and 806R (5′-GGAC TACHVHHHTWTCTAAT) primers targeting the V4 region of the 16S rRNA gene (Caporaso et al. 2011). Samples underwent PCR, in triplicate 25 µl reactions, using a Qiagen Multiplex PCR kit with the following thermocycler conditions: 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). We pooled triplicate reactions after confirming amplification success through gel electrophoresis, and then dual-indexed samples using the Nextera UD Index Kit (Ilumina, San Diego, California) and then purified with OMEGA Bio-Tek MagBind magnetic beads (Norcross, Georgia). Laragen (Culver City, California) performed quantification and pooling to create libraries with equimolar sample concentrations. Multiplexed libraries were paired-end sequenced (300 bp per sequence) on an Ilumina Miseq v3 at Laragen. We carried negative controls from the DNA extraction process and subsequent PCRs throughout sample processing and added these to the final pooled library for sequencing.

Sequencing the 207 samples yielded a total of 10,930,721 raw sequencing reads. After cleaning and filtering, 2,449,899 reads remained and were merged into a feature table for analysis. Sample sequencing depth ranged from 27 reads to 71,502 reads. As such, we rarefied all samples to a minimum depth of 1,000 reads, excluding a total of 6 samples with fewer than 1,000 reads from subsequent analysis, leaving a total of 201 samples representing 71 individuals across 5 years (2015 to 2019).

Data quality control and analysis

The resulting sequence libraries were run through the QIIME2 (v. 2019.9) microbiome data science platform (Bolyen et al. 2019) for quality control, amplicon sequence variant (ASV) taxonomy assignment, and community diversity analyses. Data were demultiplexed and denoised using "dada2" (Callahan et al. 2016) and merged into a feature table for analysis. We then rarefied samples to a minimum sequencing depth of 1,000 reads—all samples with fewer than 1,000 reads were excluded from analysis, resulting in a sample size of 201 for downstream analyses. ASVs were assigned taxonomy using a naive Bayes taxonomy classifier trained on the SILVA database (Quast et al. 2013; Yilmaz et al. 2014; Glöckner et al. 2017) with reference sequences clustering at 99% similarity. ASVs with fewer than 5 reads were pruned as well as ASVs occurring in less than 3% of the samples (Karstens et al. 2019). Any ASVs associated with assignments to eukaryotes, chloroplasts, and cyanobacterial reads were also pruned. ASVs were compiled into a table and analyzed in R version 3.5.1. (R Core Team 2014) using the package "phyloseq" (McMurdie and Holmes 2013).

To analyze the beta diversity across samples, we used the R package "vegan" (Oksansen et al., 2022) and distance matrices derived from "QIIME2." Using "vegan," we visualized samples via PCOA plots to detect significant clusters across age and colony. Clustering magnitudes were determined via PERMANOVA (999 iterations) tests for both variables.

Estimating mass gain rate

Because marmot mass gain rates vary with age (Armitage et al. 1976), repeated measures of body mass were taken for all individuals captured from 2015 to 2019. Using methods from Heissenberger et al. (2020), we used body mass at emergence from the natal burrow for juveniles, predicted 1 June body mass for yearlings and adults, and 15 August body mass for all ages—dates that reflect the bulk of the growing season for each respective age class. Predicted values were calculated by fitting a linear mixed-effects model on body mass measurements, where individual identity, year, and site were included as random effects and colony, age, and sex were fixed effects. This permitted us to generate Best Linear Unbiased Predictions for predicting 1 June and 15 August body mass (Ozgul et al. 2010; Maldonado-Chaparro et al. 2015; Heissenberger et al. 2020). We calculated juvenile growth rate as the difference from the 15 August body mass and the mass at first natal emergence divided by the number of days between them. For yearlings and adults, it was the difference between 15 August and 1 June masses divided by the number of days between them (76 days). While it was possible to use actual mass measurements rather than predicted values, it would be logistically impossible to weigh all the marmots on the same day or even a small range of days during the field season. All marmots are trapped opportunistically and we are unable to capture a given individual at will.

Testing bacterial composition influence on mass gain

We fitted linear mixed-effects models (Bates et al. 2015) to explain variation in mass gain rates. To account for limitations in sequencing and to control for spurious correlations, the phyla OTU tables were transformed using the centered log ratio, or CLR (Aitchison 1982; Gloor et al. 2017). Models included the fixed effects of CLR-transformed ASV counts assigned to Bacteroidetes or Firmicutes, and subsequent families of interest within those phyla (these continuous variables were zero and centered), valley position, and age class, and the interactions between bacterial phyla or family and valley position and bacterial phyla or family and age class. Because Ruminococcaceae and Muribaculaceae were the only families found to be significantly correlated with mass gain, we report the correlations for only these two family groups. We included year and marmot ID as random effects. We included valley position as a fixed effect, because snow melts later at the higher-elevation sites and this potential for an effect of elevation on mass gain, combined with later marmot emergence means that they live in a relatively harsher environment with less time to gain mass (Van Vuren and Armitage 1991; Blumstein 2009; Armitage 2014). We removed nonsignificant interactions and refitted the models for final interpretation (Engqvist 2005). We then estimated the marginal and conditional R^2 values using the package "MuMIn" (Bartoń 2015). Lastly, we estimated the relative amount of variation explained by the bacterial taxa by removing either the bacterial taxa or the significant interaction between the bacterial

taxa and another fixed effect, and refitting the final model without it. We set our alpha to 0.05 and consider results with $P < 0.05$ as significant. Model assumptions were evaluated by plotting their residuals (they were approximately normal), plotting q–q plots (they were roughly straight), and plotting residuals versus fitted values (there were no obvious patterns).

Results

Bacterial taxonomic composition of the marmot gut microbiome

At the phylum level, Firmicutes dominated marmot gut microbiomes, averaging 61% abundance across all samples. Bacteroidetes was the second most dominant group, averaging 29% followed by Tenericutes with an average of 6% across all samples. Actinobacteria, Proteobacteria, and Verrucomicrobia occupied the rest of most samples; the presence of other groups was less than 1% (Fig. 1A). Examining microbial abundance at the family level showed that Ruminococcaceae was the most dominant with an average abundance of 35%, followed by Lachnospiraceae with 15%, Muribaculaceae with 12%, and Rikenellaceae with 8.6% mean abundance across all samples. Rikenellaceae, Bacteroidaceae, Christensenellaceae, Clostridiales vadinBB60 group, Anaeroplasmataceae, and Erysipelotrichaceae occupied the rest of most samples (Fig. 1B). Ruminococcaceae and Lachnospiraceae families are members of the phylum Firmicutes, while Muribaculaceae and Rikenellaceae families are part of the Bacteroidetes phylum.

Gut microbes contribute to variation in marmot mass gain rates

Mass gain rates across all 71 individuals varied by age class. Adults gained mass at an average of 15.29 g/day, yearlings 21.08 g/day, and juveniles 24.53 g/day. The distribution of mass gain rates conformed to normal expectations ($W = 0.99185$, $P = 0.3223$) and therefore was not transformed. After controlling for variation explained by age class and valley position as fixed effects, and year and individual identity as random effects, we found that abundance of Firmicutes was positively associated with variation in mass gain rates (Fig. 2; Table 2; estimate = 0.645 ± 0.237 SEM, $P = 0.007$, estimated partial $R^2 = 0.011$).

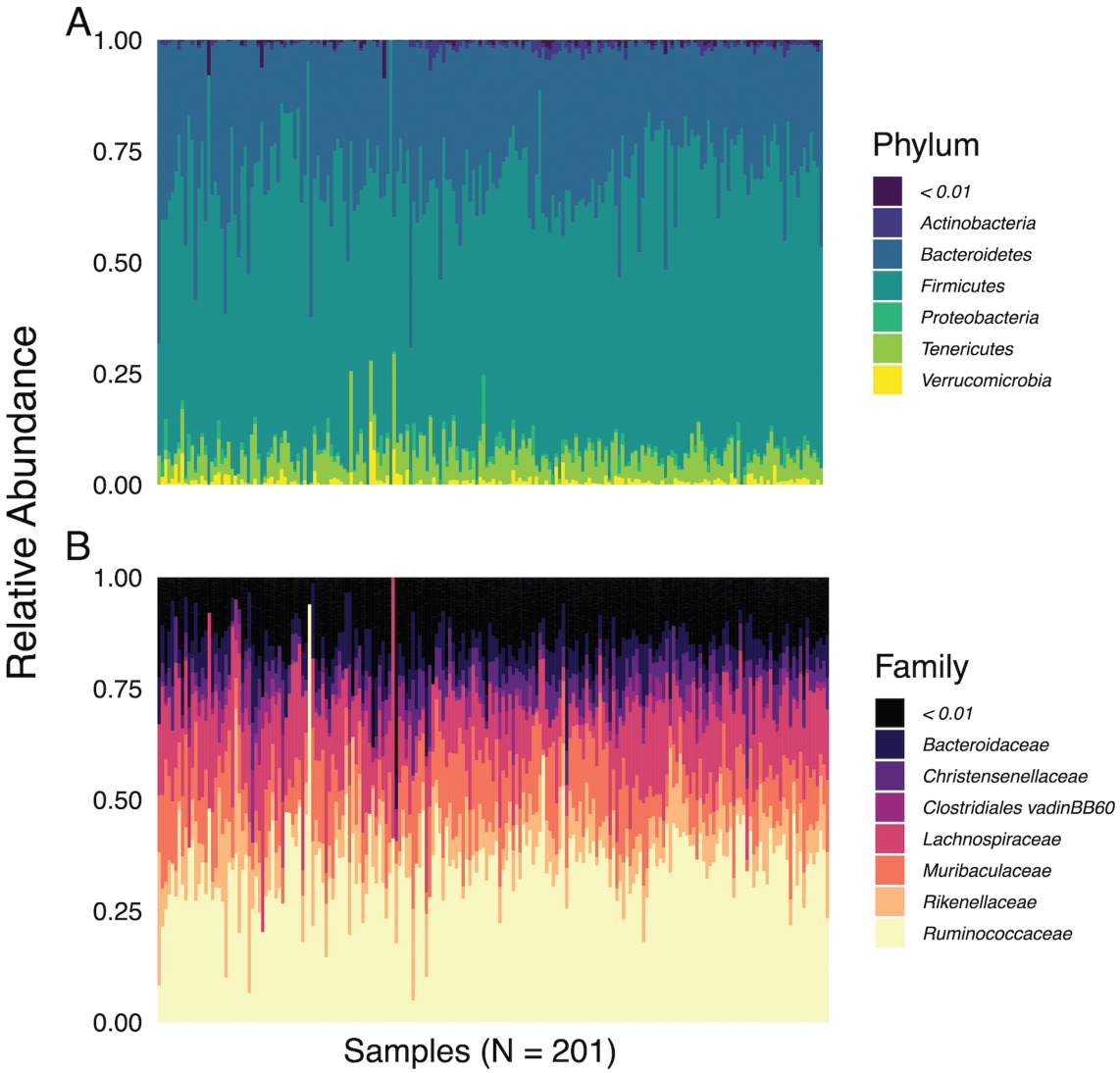

Fig. 1. A) The relative abundance of dominant gut phyla across all samples (n = 201) showing Firmicutes and Bacteroidetes occupying the majority of reads. B) The relative abundance of dominant gut families across all samples (*n* = 201).

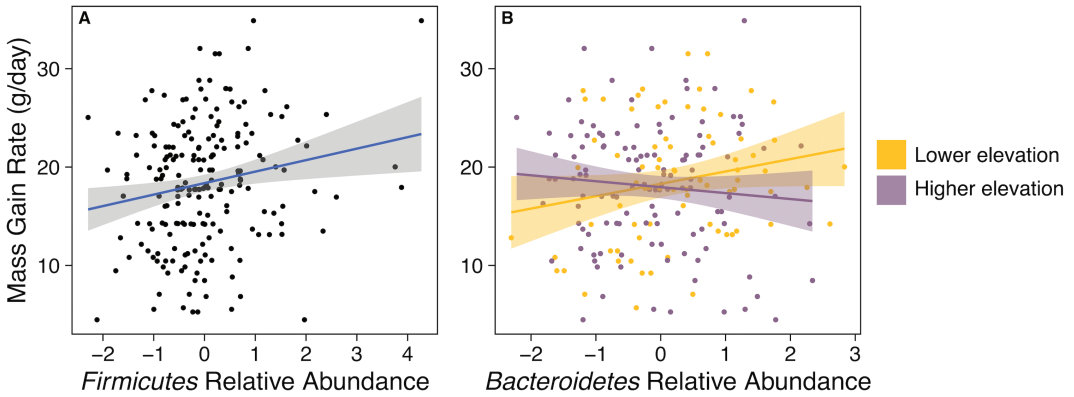

Fig. 2. A) Relationship between Firmicutes abundance and mass gain rate for all samples (n = 201) across the marmot active season. The purple line shows the predicted relationship based on the linear mixed-effects model. B) Relationship between Bacteroidetes relative abundance and mass gain rate for all samples (*n* = 201) across the marmot active season. The purple line shows the predicted relationship based on the linear mixed-effects model between Bacteroidetes relative abundance mass gain rate in higher-elevation colonies, while the orange line shows the predicted relationship between Bacteroidetes relative abundance and mass gain rate in lower-elevation colonies. Abundance measures are transformed using the centered log ratios. Shading represents 95% confidence intervals.

Table 2. Fixed and random effects from the best-fit model showing Firmicutes and age class explain variation in mass gain rates. The adult age class is used as the reference category. Significant effects are shown in bold.

Variable	Estimate (SE)	t	P
(Intercept)	**14.693**	**10.107**	**$1.9e^{-07}$**
Firmicutes	**0.645**	**2.712**	**0.007**
Valley position	1.601	1.437	0.155
Age class (J)	**−3.505**	**−3.101**	**0.002**
Age class (Y)	**4.499**	**6.749**	**$1.67e^{-10}$**

Random effects		
Groups name	**Variance**	**SD**
Marmot ID	18.229	4.270
Year	5.464	2.337
Observations	201	
Marginal R^2/conditional R^2	0.175/0.831	

Bacteroidetes was negatively associated with variation in mass gain rates, and only in higher-elevation up-valley colonies (Fig. 3; Table 3; estimate = −0.974 ± 0.432 SEM, *P* = 0.026, estimated partial R^2 = 0.039).

Age class was included as a fixed effect in each mixed model, and was significantly associated with mass gain rates for both juveniles (Table 2; Firmicutes, estimate = −3.505 ± 1.174 SEM, *P* = 0.002); (Table 3; Bacteroidetes, estimate = −3.158 ± 1.188 SEM, *P* = 0.006) and for yearlings (Table 2; estimate = 4.499 ± 0.681 SEM, *P* = 1.67×10^{-10}); (Table 3; estimate = 4.380 ± 0.679 SEM; *P* = 4.24×10^{-10}). Age class explained much of the variation in mass gain rates in both the Firmicutes model (estimated partial R^2 = 0.159) and the Bacteroidetes model (estimated partial R^2 = 0.153). Adults were used as the reference age class. Valley position did not explain variation in mass gain rates in either of the two models (Table 2; estimate = 1.601 ± 1.209 SEM, *P* = 0.155); (Table 3; estimate = 1.167 ± 1.204 SEM, *P* = 0.296).

Further analysis at the family level revealed that abundance of Ruminococcaceae within the phylum Firmicutes, and Muribaculaceae within the phylum Bacteroidetes significantly explained variation in mass gain rates. After controlling for variation explained by age class and valley position as fixed effects, and year and individual identity as random effects, we found that abundance of Ruminococcaceae was positively associated with variation in mass gain rates (Fig. 3A; Table 4; estimate = 0.528 ± 1.496 SEM, *P* = 0.016, estimated partial R^2 = 0.017). Muribaculaceae was negatively associated with variation in mass gain rates although with the opposite relationship, and only in up-valley colonies (Fig. 3B; Table 5; estimate = −1.416 ± 0.531 SEM, *P* = 0.008, estimated partial R^2 = 0.024).

Age class was also associated with mass gain rates at the family level analysis for both juveniles (Table 4; Ruminococcaceae, estimate = 5.835 ± 1.177 SEM, $P = 1.68 \times 10^{-6}$); (Table 5; Muribaculaceae, estimate = 6.098 ± 1.176 SEM, $P = 6.02 \times 10^{-7}$) and for yearlings (Table 4; estimate = 4.284 ± 0.680 SEM, $P = 2.00 \times 10^{-9}$); (Table 5; estimate = 4.357 ± 0.676 SEM, $P = 8.88 \times 10^{-10}$). Age class also explained much of the variation in mass gain rates in both the Ruminococcaceae model (estimated partial R^2 = 0.155) and the Muribaculaceae model (estimated partial R^2 = 0.157). Adults were used as the reference age class. Valley position did not explain variation in mass gain rates in either of the two models (Table 4; estimate = 1.265 ± 1.216 SEM, *P* = 0.301); (Table 5; estimate = 1.123 ± 1.204 SEM, *P* = 0.353).

Microbial composition is not influenced by age class or valley position

Individuals within the same age class or living in the same part of the valley (either at higher- or lower-elevation sites) did not cluster by gut microbiome composition similarities even when considering only Firmicutes and Bacteroidetes. Principle coordinate analysis (PCoA) of Bray–Curtis distance metrics on the overall composition of the marmot fecal microbiome revealed no pattern of clustering between different age classes or individuals living up- or down-valley. *PERMANOVA* analysis confirmed no significant pattern across age classes (*P* = 0.162) or valley position (*P* = 0.490; Fig. 4).

Discussion

Our comparison of microbiome composition showed that the relative abundance of Firmicutes and Bacteroidetes was significantly associated with mass gain in marmots, as reported for other

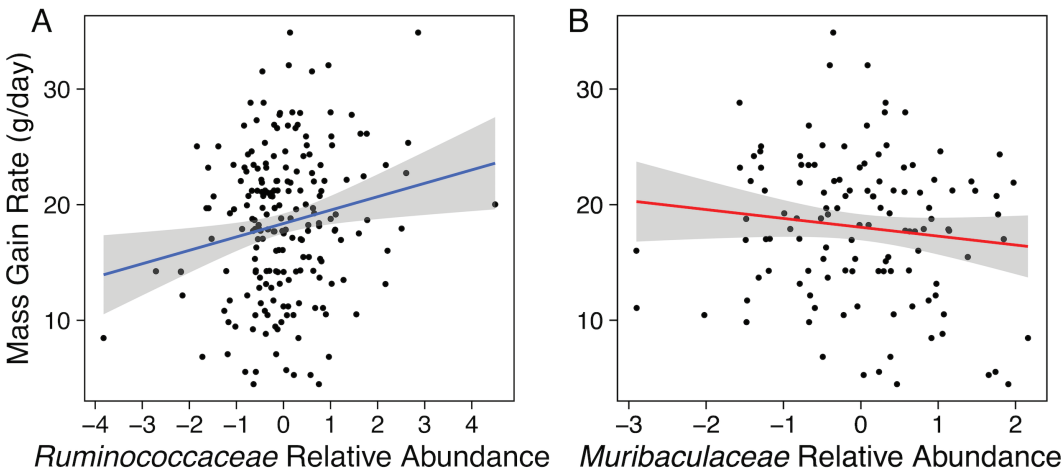

Fig. 3. A) Relationship between Ruminococcaceae relative abundance and mass gain rate for all samples (n = 201) across the marmot active season. The line shows the predicted relationship based on the linear mixed-effects model. B) Relationship between Muribaculaceae relative abundance and mass gain rate for only up-valley samples (n = 122) across the marmot active season. The line shows the predicted relationship based on the linear mixed-effects model between Muribaculaceae relative abundance mass gain rate in higher-elevation colonies. Gray shading represents 95% confidence intervals.

Table 3. Fixed and random effects from the best-fit model showing Bacteroidetes in up-valley colonies (UV) and age class explaining variation in mass gain rates. The adult age class is used as the reference category. Significant effects are shown in bold.

Variable	Estimate (SE)	t	P
(Intercept)	**15.008**	**10.675**	**3.98e^{-08}**
Bacteroidetes	0.092	0.293	0.770
Valley position (UV)	1.167	1.052	0.296
Age class (J)	**−3.158**	**−2.763**	**0.006**
Age class (Y)	**4.380**	**6.581**	**4.24e^{-10}**
Bacteroidetes × UV	**−0.975**	**−2.253**	**0.026**

Random effects		
Groups name	**Variance**	**SD**
Marmot ID	18.406	4.290
Year	4.878	2.209
Observations	201	
Marginal R^2/conditional R^2	0.172/0.829	

Table 4. Fixed and random effects from the best-fit model showing Ruminococcaceae and age class explain variation in mass gain rates. The adult age class is used as the reference category. Significant effects are shown in bold.

Variable	Estimate (SE)	t	P
(Intercept)	**14.989**	**10.017**	**4.68e^{-08}**
Ruminococcaceae	**0.528**	**2.245**	**0.016**
Valley position	1.265	1.040	0.301
Age class (J)	**5.835**	**4.956**	**1.68e^{-06}**
Age class (Y)	**4.284**	**6.293**	**2.00e^{-09}**

Random effects		
Groups name	**Variance**	**SD**
Marmot ID	18.550	4.307
Year	5.079	2.466
Observations	201	
Marginal R^2/conditional R^2	0.170/0.830	

mammals (Ley et al. 2005; Turnbaugh and Gordon 2009; Crovesy et al. 2020; Stojanov et al. 2020). To our knowledge, this is the first study to investigate the effect of gut microbes on mass gain in a hibernating animal from a wild population. Other studies on microbiomes and mass gain remove animals from their native habitat, potentially altering the microbiome due to changes in diet, environmental factors, and interactions with humans during captivity (Uenishi et al. 2007; Dhanasiri et al. 2011; Clayton et al. 2016). Moreover, the few gut microbiome studies on hibernating species found no significant effect of gut microbe abundance on seasonal fattening (Stevenson et al. 2014a; Sommer et al. 2016), a result that may result from low sample sizes (n = 46 and n = 16) necessitated by keeping animals in captivity. By examining wild populations, with a much larger sample size (n = 201), our study was able to detect a significant impact of microbiome composition on mass gain.

Like previous studies (Ley et al. 2005; Turnbaugh and Gordon 2009; Crovesy et al. 2020; Stojanov et al. 2020), our results from marmots showed that relative abundances of *Firmicutes* and Bacteroidetes were significantly associated with mass gain. In Firmicutes, we found that the family Ruminococcaceae explained a significant portion of the association between Firmicutes and higher mass gain rates in marmots. Ruminococcaceae is a well-studied bacterial family comprised of microbial taxa critical to gut fermentation of indigestible fibers for mammalian ruminants (Malmuthuge and Guan 2016; Xie et al. 2016), and has been previously reported in the gut microbiomes of Arctic ground squirrels (Stevenson et al. 2014a). Ruminococcaceae species produce short-chain fatty acids in the gut of mammals, which in turn provide net energy gains for the host (Xie et al. 2016). Our results suggest that Ruminococcaceae are key players in marmot mass gain, further supporting observations that Ruminococcaceae species are vital symbionts for herbivorous mammals (La Reau and Suen 2018). Marmots are generalist herbivores (Frase and Armitage 1989) and would likely benefit

Table 5. Fixed and random effects from the best-fit model showing Muribaculaceae in up-valley colonies (UV) and age class explaining variation in mass gain rates. The adult age class is used as the reference category. Significant effects are shown in bold.

Variable	Estimate (SE)	t	P
(Intercept)	**15.201**	**10.286**	**2.62e^{-08}**
Muribaculaceae	−0.059	−0.242	0.809
Valley position (UV)	1.123	0.933	0.353
Age class (J)	**6.098**	**5.182**	**6.02e^{-07}**
Age class (Y)	**4.357**	**6.445**	**8.88e^{-10}**
Muribaculaceae × UV	**−1.416**	**−2.667**	**0.008**

Random effects		
Groups name	**Variance**	**SD**
Marmot ID	18.361	4.285
Year	4.928	2.446
Observations	201	
Marginal R^2/conditional R^2	0.177/0.831	

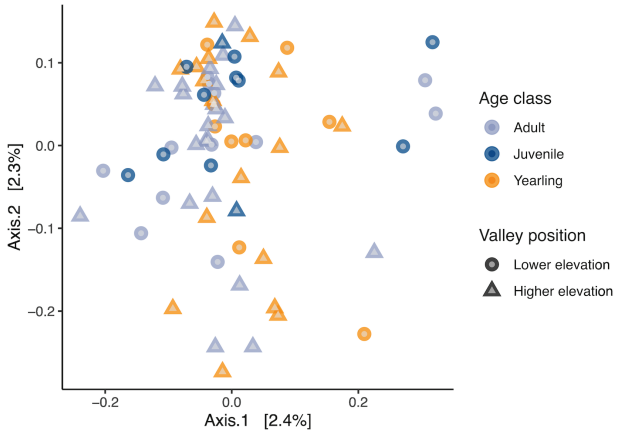

Fig. 4. Principle coordinate analysis of Bray–Curtis distance metric of gut microbiomes from 71 unique individual marmots. Each point represents an individual marmot gut microbiome, while color is assigned to the 3 different age classes and shape is assigned to valley position (up- and down-valley). For visualization purposes, samples from the same individual from different time points were merged.

from the fiber-degrading properties of Ruminococcaceae species. While other indirect effects from shifts in Firmicutes and Ruminococcaceae species may influence mass gain, such as immune function, further research is needed to explore these possibilities.

Within Bacteroidetes, Muribaculaceae explained a significant portion of the association between *Bacteroidetes* and lower mass gain rates in marmots. While Muribaculaceae species are largely involved in carbohydrate degradation (Lagkouvardos et al. 2019), they are negatively associated with obesity in lab mice (Lagkouvardos et al. 2016; Barouei et al. 2017; Obanda et al. 2018). Obanda et al. (2018) demonstrated that obese-prone mice did not gain any weight despite increased levels of Muribaculaceae species, suggesting that carbohydrate degradation performed by Muribaculaceae species does not provide a net energy gain to the host. Thus, there may be other associations of Muribaculaceae species with other physiological processes within the host, including amino acid degradation and kidney function (Barouei et al. 2017), which may result in energy deficiencies that counteract the energetic benefits of carbohydrate degradation.

While the environment of an animal can directly influence phenotype, such as seen in the white coats of snowshoe hares (*Lepus americanus*) and some other mammals during winter months, bacterial symbionts also influence phenotype (Shreiner et al. 2015; Broom and Kogut 2018; Lynch and Hsiao 2019). Therefore, the host–microbiome–environment relationship can be complex and vary across individuals that live in different places (Koskella and Bergelson 2020). In this study, the higher relative abundance of Bacteroidetes/Muribaculaceae was associated with lower mass gain rates only in animals from higher-elevation colonies. Assuming Bacteroidetes species leads to lower mass gain, it could be that marmots that live in lower elevation and less harsh conditions are less likely to be influenced by variation in Bacteroidetes because they are able to offset the cost of having more Bacteroidetes by eating and gaining mass for a longer period of time. Snow melts at our lower-elevation sites about 2 weeks earlier than our higher-elevation sites leading to an extended growing season (Van Vuren and Armitage 1991; Blumstein 2009; Armitage 2014). Therefore, animals living in harsher environments may be more effected by the abundance of Bacteroidetes than those living in less harsh conditions—even a few days variation in the time of emergence can be the difference between survival and death (Armitage 1976; Van Vuren and Armitage 1991).

It is important to note that the effect of Bacteroidetes and Firmicutes, and the families within those phyla (Ruminococcaceae and Muribaculaceae) was comparatively small compared to other factors including age. In addition to age, valley position and environmental conditions also explain variation in overwinter survival (Van Vuren and Armitage 1991; Armitage 2014). Given our prior research on marmots showing that age and sex explain much of the variation in mass gain and ability to fatten prior to hibernation, we expected the effects of microbes to be relatively small.

Interestingly, our results show no patterns of similarities or clustering of gut microbiomes between the different age classes or animals living at different elevations. Lack of clustering between age classes may be due to the social behavior of this species (Armitage 1991), because regardless of age, all animals in groups within colonies are sharing the same burrow and consistently interacting with one another (Blumstein et al. 2004), and social behavior has been shown to be a direct mode for microbial transmission (Archie and Tung 2015; Sarkar et al. 2020a). Additionally, studies have shown a positive relationship between frequency of interactions of individuals in social species and similarity of their microbiomes (Moeller et al. 2016). Lack of clustering by colony elevation may be explained by similar diets in each region. The furthest distance between up- and down-valley colonies is 4.9 km and vegetation types are essentially identical. It is expected that with increasing physical distance between hosts, beta diversity between hosts or groups would increase because microbial transmission is attenuated (Moeller et al. 2017). Although dispersal between up-valley and down-valley sites is relatively uncommon, animals occupy the same valley, and are therefore not geographically separated (Armitage 1991). Thus, our results demonstrate that the marmot gut microbiome is considerably stable across host age and environment.

The host–microbiota symbiosis is likely an important component of the hibernation phenotype (Regan et al. 2022). Given that marmots are obligate hibernators and fat accumulation is an indicator of good body condition and directly related to fitness in the wild (Haramis et al. 1986; Tammaru et al. 1996; Merilä and Svensson 1997; Festa-Bianchet et al. 2011), gut microbiome compositions of marmots that induce weight gain should confer greater survival and reproductive success, at least in adults (Jebb et al. 2021). Our results are consistent with human studies that show an association between Firmicutes, Bacteroidetes, and obesity (Ley et al. 2005, 2006; Abdallah Ismail et al. 2011; Koliada et al. 2017). Furthermore, hibernating brown bears exhibit higher relative abundances of Bacteroidetes and lower relative abundances of Firmicutes, whereas active bears trend in the opposite direction (Sommer et al. 2016), adding further support that these two phyla are critical players in body condition modulation. While we acknowledge that these associations have been brought into question in other human studies (Indiani et al. 2018; Crovesy et al. 2020), our study shows that Firmicutes and Bacteroidetes are indeed correlated with mass gain, particularly when examining a large sample size of gut microbiomes from wild hibernating hosts. Gaining insight into how animals are affected by their resident microbes can help us understand unique adaptations to harsh conditions and a changing dietary landscape and will provide essential information to future conservation and management planning (Carthey et al. 2020; Mueller et al. 2020), and applications for human and animal health. Symbiotic microbes are important to animals and humans alike, and investigating this relationship in an evolutionary context is of great interest and importance.

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Author contributions

The study was designed by GCJ, PHB, and DTB. GCJ and DTB collected the data. GCJ and SD analyzed the data. GCJ, DTB, and SD interpreted the results. GCJ wrote the initial draft of manuscript, with contributions and critical revisions by SD, PHB, and DTB.

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Conflict of interest

None declared.

Data availability

All raw sequences associated with this project are uploaded to the SRA Database with the Project ID: PRJNA885144. Data and scripts used for analyses are available at https://github.com/samd1993/marmot.

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