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ANIMAL HOST–MICROBE INTERACTIONS

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Gut microbial communities of American pikas (*Ochotona princeps*): Evidence for phylosymbiosis and adaptations to novel diets

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Abstract

1. Gut microbial communities provide many physiological functions to their hosts, especially in herbivorous animals. We still lack an understanding of how these microbial communities are structured across hosts in nature, especially within a given host species. Studies on laboratory mice have demonstrated that host genetics can influence microbial community structure, but that diet can overwhelm these genetic effects.
2. We aimed to test these ideas in a natural system, the American pika (*Ochotona princeps*). First, pikas are high-elevation specialists with significant population structure across various mountain ranges in the USA, allowing us to investigate whether similarities in microbial communities match host genetic differences. Additionally, pikas are herbivorous, with some populations exhibiting remarkable dietary plasticity and consuming high levels of moss, which is exceptionally high in fibre and low in protein. This allows us to investigate adaptations to an herbivorous diet, as well as to the especially challenging diet of moss.
3. Here, we inventoried the microbial communities of pika caecal pellets from various populations using 16S rRNA sequencing to investigate structuring of microbial communities across various populations with different natural diets.
4. Microbial communities varied significantly across populations, and differences in microbial community structure were congruent with genetic differences in host population structure, a pattern known as "phylosymbiosis."
5. Several microbial members (*Ruminococcus*, *Prevotella*, *Oxalobacter* and *Coprococcus*) were detected across all samples, and thus likely represent a "core microbiome." These genera are known to perform a number of services for herbivorous hosts such as fibre fermentation and the degradation of plant defensive compounds, and thus are likely important for herbivory in pikas. Moreover, pikas that feed on moss harboured microbial communities highly enriched in Melainabacteria. This uncultivable candidate phylum has been proposed to ferment fibre for herbivores, and thus may contribute to the ability of some pika populations to consume high amounts of moss.
6. These findings demonstrate that both host genetics and diet can influence the microbial communities of the American pika. These animals may be novel sources of fibre-degrading microbes. Last, we discuss the implications of population-specific microbial communities for conservation efforts in this species.

KEYWORDS
gut microbiota, host–microbe interactions, phylosymbiosis, pika

1 | INTRODUCTION

Associations between complex communities of gut microbes and animal hosts can have profound influences on ecology and evolution (McFall-Ngai et al., 2013). For instance, microbial communities can affect host nutrition, immune function, and even behaviour (Ezenwa, Gerardo, Inouye, Medina, & Xavier, 2012; Kohl & Carey, 2016; McFall-Ngai et al., 2013). However, we still lack a thorough understanding of the determinants of microbial community structure across diverse animal hosts, especially in natural systems.

Within a host species, populations may exhibit differential microbial communities as a result of a numerous factors. Environmental variables such as habitat degradation (Amato et al., 2013), temperature (Chevalier et al., 2015) or parasite infections (McKenney et al., 2015) can alter the community structure of the gut microbiota. Moreover, there can be host genetic effects on gut microbial community structure. In an evolutionary sense, host genetics may contribute to microbial community structure in such a way that more closely related species would harbour more similar gut microbial communities (Brucker & Bordenstein, 2012a, 2012b). This congruence between host evolutionary history and similarities in microbial communities has been termed "phylosymbiosis" (Brucker & Bordenstein, 2012a, 2012b). While phylosymbiosis has been demonstrated across host species under laboratory-controlled settings (Brooks, Kohl, Brucker, van Opstal, & Bordenstein, 2016), its prevalence among animals under natural conditions is unclear and has not been investigated across populations of the same species.

One of the strongest determinants of gut microbial community structure is diet. In mice, dietary effects overwhelm genetic effects in sculpting microbial diversity (Carmody et al., 2015). Across mammalian feeding groups, herbivores, omnivores and carnivores harbour distinct microbial communities with specialized functions for their given diets (Ley et al., 2008; Muegge et al., 2011). Associations with gut microbes are particularly important for mammalian herbivores, thereby allowing them to consume high-fibre plant material often laden with toxic compounds (Kohl, Weiss, Cox, Dale, & Dearing, 2014; Mackie, 2002). Understanding the specific microbial taxa involved in permitting herbivores to consume novel food sources has been well studied in agricultural ruminants, but far less so in other free-living species.

The American pika (*Ochotona princeps*) offers a unique study system in which to study inter-population variation in gut microbial communities, as well as adaptations to herbivorous diets. Pikas are high-elevation, alpine specialists, resulting in isolated "sky island" populations, with limited dispersal and population mixing (Galbreath, Hafner, & Zamudio, 2009). As a result, there is significant population structure across various mountain ranges of the Intermountain West of the USA (Galbreath et al., 2009; Hafner & Smith, 2010).

In terms of diet, pikas are herbivorous and feed largely on a variety of grasses and forbs (Dearing, 1997). Pikas collect plants from alpine meadows and store them in haypiles within barren talus fields. Through these actions, pikas alter the plant community structure of alpine meadows (Huntly, 1987) and nutrient concentrations in talus fields (Aho, Huntly, Moen, & Oksanen, 1998). In some locations, pikas exhibit remarkable dietary flexibility. For example, the diet of populations of pikas in the Columbia River Gorge of Oregon, USA is composed of roughly 60% moss (Varner & Dearing, 2014). Feeding on moss presents extreme nutritive challenges, as it contains roughly double the fibre and significantly less nitrogen than typical food sources (Varner & Dearing, 2014). Remarkably, pikas in the Columbia River Gorge exhibit the highest moss intakes documented for any wild mammalian species (Varner & Dearing, 2014). Thus, populations of pikas in the Columbia River Gorge may harbour distinct microbial communities that help them to cope with these nutritive challenges.

We inventoried the gut microbial communities of several populations of American pikas. With these data, we asked several questions. (i) How do microbial communities differ across pika populations? Also, given that the biogeographic relationships across these populations have been well studied, is there "phylosymbiosis" between host phylogeny and similarities in gut microbial communities? (ii) Which microbial members are present across pika populations, and thus may represent a "core microbiota?" (iii) Which microbes are enriched in the moss-feeding pika population, and thus may represent an adaptation to this difficult diet? Both core microbial members and those enriched in the moss-feeding pikas may give insight into these animals' abilities to feed on fibre-rich, protein-deficient food sources.

2 | MATERIALS AND METHODS

2.1 | Sample collection

Like all lagomorphs, pikas are coprophagic and produce two types of scat or droppings. Faecal pellets are hard, round and contain largely undigested plant material and are not reingested, whereas caecal pellets are soft, amorphous and contain the highly nutritious products of microbial fermentation in the caecum, and thus are often reingested by the animal (Smith & Weston, 1990). Pika caecal pellets contain approximately six times more nitrogen and less than half the fibre compared to the plants that the pikas consume (Varner & Dearing, 2014). Unlike most lagomorphs, which void caecal pellets at night and consume them directly, pikas produce caecal pellets throughout the day, and may either consume them immediately or cache them in haypiles for later consumption (Smith & Weston, 1990).

A total of 55 fresh caecal pellets were collected from individual pikas in five populations: the Wasatch mountains of northern Utah (n = 6 samples), the Gallatin range of western Montana (n = 3 samples),

the Front range of Colorado (n = 5 samples), and two populations in northern Oregon, where we sampled at Mt. Hood (n = 21 samples) where pikas feed on typical food resources, and in the Columbia River Gorge (n = 20 samples) where pikas consume high amounts of moss. In Colorado, Montana and Oregon, pikas were live-trapped and handled for other studies (Varner & Dearing, 2014; Wilkening & Ray, 2016). At these sites, fresh caecal pellets were collected directly from the animal, placed in a tube of RNAlater, and then transferred to a −80°C freezer where they were stored until DNA extraction.

To increase our sample size in Oregon and include the Utah population, we obtained additional fresh caecal pellets from active haypiles at these sites. Fresh pellets in haypiles were identified by colour (typically dark green) and consistency (wet, without a hard, dark crust). In our experience, caecal pellets with these characteristics were likely to have been deposited within a few hours. Only one sample was collected from each occupied territory, ensuring that these samples came from independent animals. These pellets were collected with sterile tweezers into a tube of RNAlater (Ambion, Foster City, CA, USA), then stored at −80°C until DNA was extracted.

2.2 | Sequencing and data analysis

Total DNA was isolated from caecal pellets using a QIAamp DNA Stool Mini Kit (Qiagen, Germantown, MD, USA). Extracted DNA was sent to Argonne National Laboratory for sequencing. The primers *515F* and *806R* were used to amplify the V4 region of the 16S rRNA gene (Caporaso et al., 2012). Amplicons were sequenced on the Illumina MiSeq platform using previously described techniques (Caporaso et al., 2012).

Microbial sequences were analysed using the QIIME version 1.9.1 (Caporaso et al., 2010). We applied standard quality control settings and split sequences into libraries using default parameters in QIIME. Sequences were grouped into de novo operational taxonomic units (OTUs) using a minimum sequence identity of 97% (He et al., 2015). The most abundant sequences within each OTU were designated as a "representative sequence" and aligned against the Greengenes core set (DeSantis et al., 2006) using PyNAST (Caporaso et al., 2009) with default parameters set by QIIME. FastTree (Price, Dehal, & Arkin, 2009) was used to generate a phylogenetic tree of representative sequences. Taxonomic classification of OTUs was performed using UCLUST (Edgar, 2010). Singleton OTUs and sequences identified as chloroplasts or mitochondria were removed from the analysis.

We compared several aspects of gut microbial community diversity and structure. First, we compared microbial community structure by calculating Bray–Curtis distances (Beals, 1984) using 19,500 sequences per sample and conducting principal coordinates analysis (PCoA). Whether populations significantly differed in community structure was determined using the ANOSIM test in QIIME (Anderson, 2001).

To investigate similarities in microbial community structure across mountain ranges, we first pooled sequences within each population. Then, we used the jackknifed_beta_diversity.py script in QIIME to generate UPGMA trees (Unweighted Pair Group Method with Arithmetic Mean) using 146,000 sequences from each of the pooled microbial communities from each mountain range. This tree was compared to the phylogeny of pika populations as determined previously (Galbreath et al., 2009).

We compared the relative abundances of microbial taxa across mountain ranges. Relative abundances of phyla and genera in each individual sample were transformed using a variance stabilizing transformation of arcsin(abundance$^{0.5}$) (Kumar, Mason, Brooker, & O'Brien, 2012; Shchipkova, Nagaraja, & Kumar, 2010). Then, we used JMP®, Version 12.0 (SAS Institute Inc., Cary, NC, USA) to compare relative abundances of bacterial phyla and genera using the Response Screening function with the robust fit option to conduct multiple ANOVAs and correct p-values with the Benjamini–Hochberg false discovery rate (FDR) correction (Benjamini & Hochberg, 1995). We also conducted pairwise comparisons of microbial abundances between pika populations from different states to explore differences in abundances. For this, we conducted LEfSe (linear discriminant analysis effect size; Segata et al., 2011) with a threshold logarithmic LDA score of 2.0. Last, "core OTUs" were defined as those present in 100% of the 55 samples we analysed.

We calculated several aspects of alpha diversity for each sample (Shannon index, observed OTUs, evenness and Faith's phylogenetic diversity [Faith, 1992]) and compared diversity across populations. For each sample, we calculated the mean of 20 iterations for a subsampling of 19,500 sequences. This sequencing depth was sufficient to capture a majority of the microbial diversity (Figure S1).

We also conducted further analysis comparing the typical-feeding and moss-feeding populations in Oregon. Here, we conducted principal coordinates analysis as described above, and tested for differences in microbial community structure using the ANOSIM test. We also compared the relative abundances of microbial phyla and genera, and alpha diversity measurements as described above, with t-tests.

3 | RESULTS

Our sequencing effort returned an average of 76,604 ± 6,309 sequences per sample. There was no difference in number of sequences across populations ($F_{3,51}$ = 1.18; p = .33). Within the Oregon population, there were more than twice as many sequences from the pikas that consume typical diets (113,454 ± 12,149 per sample) compared to samples collected from the moss-feeding population (51,767 ± 3,552; t test: p < .0001). The sequences were classified into 144,222 total OTUs.

Pika populations from different mountain ranges harboured distinct gut microbial communities. A principal coordinates analysis of the Bray–Curtis distance matrix displayed the samples from different mountain ranges as clustering distinctly (Figure 1; ANOSIM: R = 0.57, p < .001). A cladogram of microbial community similarity was congruent with the host phylogeny determined by Galbreath et al., (2009), suggesting that evolutionary history plays a role in determining the structure of pika gut microbial community (Figure 2).

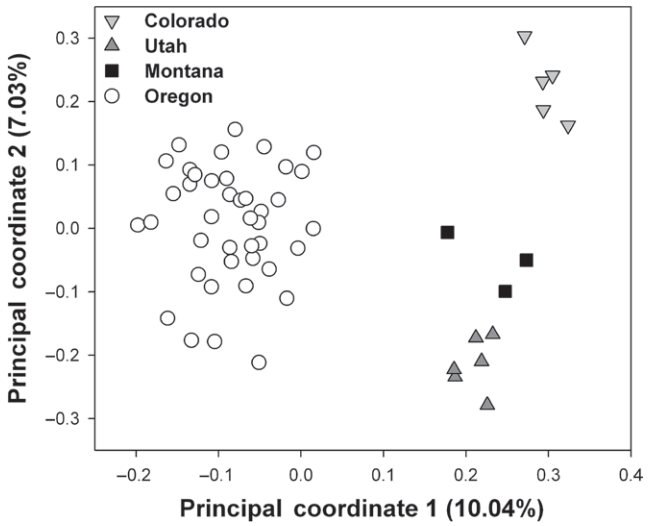

FIGURE 1 Principal coordinate analysis of pika caecal pellet communities using Bray–Curtis distances. Points that cluster together share more similar microbial communities

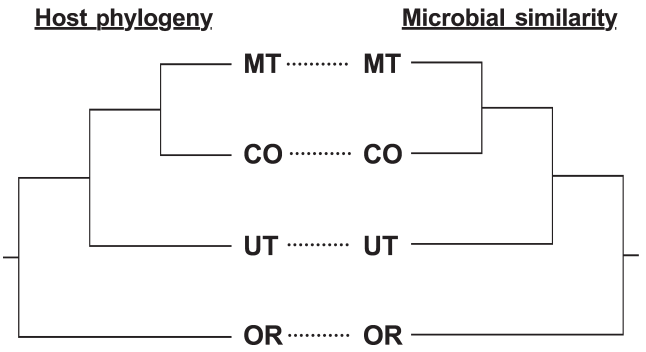

FIGURE 2 Cladograms of host phylogeny and microbial community similarity. Host phylogeny is based on previous investigations into pika genetic structure across mountain ranges (Galbreath et al., 2009). Microbial community similarity was determined by generating a UPGMA tree (Unweighted Pair Group Method with Arithmetic Mean) as described in the Materials and methods. Bootstrap values were 1 at all nodes for the microbiome cladogram

The relative abundances of seven phyla of microbes differed significantly across populations (Figure 3; Table S1). Specifically, pikas from Colorado harboured communities enriched in Tenericutes, pikas from Utah hosted communities with high abundances of Firmicutes and gut communities of pikas from Oregon were enriched in Melainabacteria. Furthermore, we identified 36 genera (36% of observed genera) that exhibited significant differences in relative abundances across populations (Table S1). Pikas from Montana hosted caecal communities especially enriched in *Prevotella*. Pairwise comparisons of states demonstrated that closely related populations (Montana and Colorado) had only three bacterial genera that were differentially abundant, while the basal population (Oregon) had 13–17 genera that were differentially abundant from other populations (Table S2). However, despite these differences in microbial abundances, the gut communities of pikas did not differ in any measurements of alpha diversity (Shannon index, observed OTUs, evenness, or Faith's phylogenetic diversity).

Although microbial community membership varied across populations, there were a number of shared microbes across all samples. There were 85 OTUs (0.06% of the total OTUs) detected in all pika caecal samples, and thus represent a core set of pika gut microbes. These OTUs were identified as *Prevotella*, *Coprococcus*, *Oscillospira*, *Ruminococcus*, *Oxalobacter*, *Campylobacter*, and several unidentified OTUs in the families S24-7, Lachnospiraceae, and Ruminococcaceae. The majority of these core OTUs were present at low abundances, composing less than 1% of the communities, although four additional OTUs had average relative abundances greater than 1% of the communities. These OTUs were identified as an unidentified member of the family Lachnospiraceae (1.4 ± 0.2% of the community), a member of the genus *Prevotella* (2.1 ± 0.2%), a member of the putative family S24-7 (2.1 ± 0.1%) and a member of the putative family RF16 (2.8 ± 0.4%).

When focusing solely on the Oregon pika populations, microbial community structure differed significantly between typical-feeding and moss-feeding populations. These populations clustered differentially in a principal coordinates analysis of the Bray–Curtis distance matrix (Figure 4; ANOSIM: $R = 0.11$, $p < .001$; a graph depicting PCo1 and PCo2 can be found as Figure S2). We identified two microbial phyla that exhibited significantly differential abundances between these Oregon pika populations. The phylum Actinobacteria composed 2.94 ± 0.03% of the gut community of typical-feeding pikas, but only 1.44 ± 0.01% of the community of moss-feeding pikas (FDR-corrected $p < .001$). Additionally, the phylum Melainabacteria was significantly more abundant in the moss-feeding pika population (Figure 4b; FDR-corrected $p < .001$). In fact, moss-feeding pikas harboured a community highly enriched in Melainabacteria when compared to the communities of pikas from the other populations in our study and to other mammalian herbivores that have been previously studied (Figure 4b). Only a single microbial genus (of 99 observed genera) differed in abundance between typical-feeding and moss-feeding pika populations. This genus, *Adlercreutzia* (Phylum Actinobacteria), was present at a relative abundance of 1.41 ± 0.02% in typical high-elevation pikas, compared to 0.73 ± 0.01% in moss-feeding pikas (FDR-corrected $p = .02$). There were no differences in any measurements of alpha diversity (Shannon index, observed OTUs, evenness or Faith's phylogenetic diversity) between typical-feeding and moss-feeding pika populations.

4 | DISCUSSION

We have limited understanding of how gut microbial communities differ across animal populations in natural environments as a result of evolution, diet or other factors. In this paper, we used microbial community inventories from pika caecal pellets to test for (i) phylosymbiosis between the genetic structure of various pika populations and their gut microbial communities and (ii) potential microbes that may assist pikas in feeding on high-fibre plant material, especially moss. These results expand our understanding of host–microbe associations and highlight the pika gut microbiota as a potential source of novel fibre-degrading microbes.

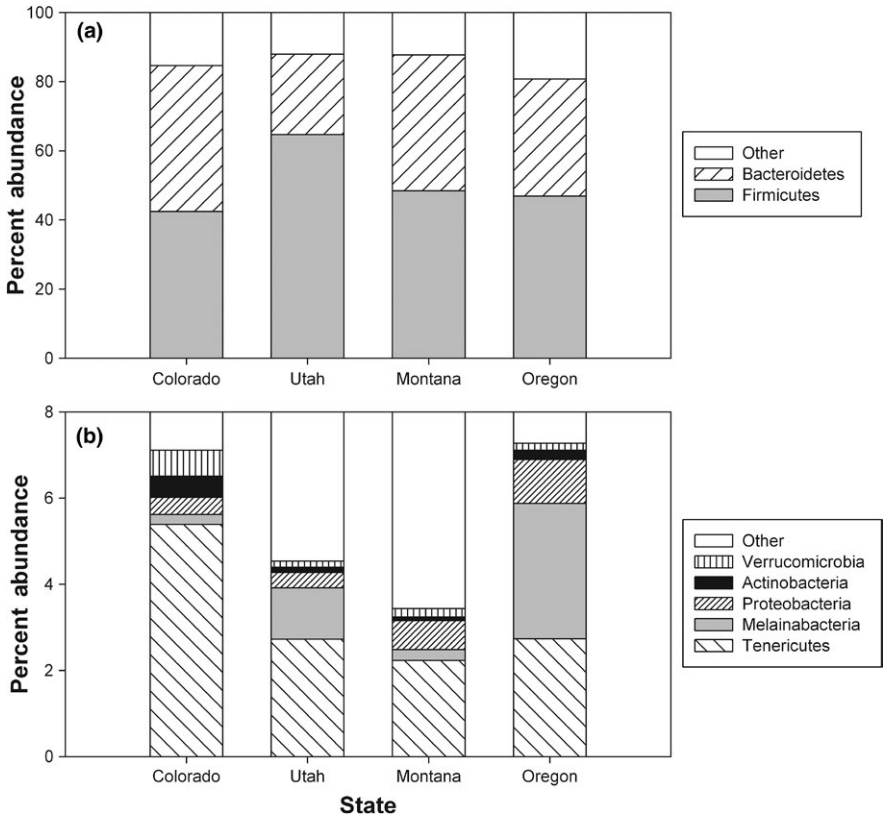

FIGURE 3 Relative abundances of microbial phyla across various populations. Panel (b) focuses on rare taxa that are represented as "Other" in panel (a)

Microbial communities varied significantly across populations from different mountain ranges. Most notably, similarities in these microbial communities were congruent with genetic relationships of these populations, indicating a phylosymbiotic relationship between pika hosts and their associated gut microbes. Patterns of phylosymbiosis have been robustly demonstrated under controlled laboratory settings (Brooks et al., 2016), but with equivocal evidence in wild systems (Baxter et al., 2015; Hird, Sánchez, Carstens, & Brumfield, 2015; Moeller et al., 2014; Ochman et al., 2010; Phillips et al., 2012). Moreover, previous studies of phylosymbiosis have investigated variations in microbial communities across various host species. Here, we investigate this pattern across distinct populations of the same host species, demonstrating that evolution may result in distinguishable microbial communities relatively quickly as populations diverge genetically. A number of mechanisms might underlie the observed pattern of phylosymbiosis, such as evolution of immune genes and host-produced glycans, or vertical transmission of microbes from mother to offspring (see [Brooks et al., 2016] for discussion). However, as our samples were collected from free-living animals, it should be noted that the observed pattern of phylosymbiosis might be driven by differences in diet, host density or a number of other environmental factors, especially as these factors have been suggested to influence the microbial communities of Asian pika species (Li, Li, Beasley et al., 2016; Li, Li, Yao et al., 2016; Li, Qu et al., 2016). Controlled feeding studies would be necessary to properly address these other drivers; unfortunately, such studies may be challenging given that American pikas do not thrive in captivity (MacArthur & Wang, 1973).

It is possible that microbial communities may differ slightly between samples collected directly from animals compared to those that had been in haypiles for several hours. For example, a controlled study documented that the microbial community structure of faeces can change over a 24-hr period under field conditions (Hale et al., 2016). However, this study used a single pooled faecal sample, and so it is unclear whether the effects of time are larger or smaller than inter-individual variation. A controlled study with multiple individuals of woodrats found that individual microbial signatures were highly retained between fresh faeces and those collected from the floor of Sherman traps after the rodent hosts spent a night in the trap with cotton batting, apple slices and oatmeal bait for ~10 hr (Kohl, Luong, & Dearing, 2015). Thus, it is unlikely that the effects of field conditions are solely responsible for the large differences in microbial community structure across populations.

Several microbial OTUs were present across all pika caecal pellets, and thus represent a "core microbiota." The taxonomic identification of several of these OTUs suggests that they may be important for the digestion of fibre-rich plant material. For example, members of the genera *Ruminococcus* and *Prevotella* are common in rumen environments and are well known for their fibre-degrading capabilities (Avgustin, Flint, & Whitehead, 1992; Leatherwood, 1965). These genera were also dominant in the guts of Daurian and Plateau pikas in China (*O. daurica* and *O. curzoniae*; Li, Li, Beasley et al., 2016; Li, Li, Yao et al., 2016). Additionally, *Coprococcus* is an abundant genus in the guts of other mammalian herbivores, such as herbivorous woodrats (Kohl, Weiss, Dale, & Dearing, 2011) and the North American beaver

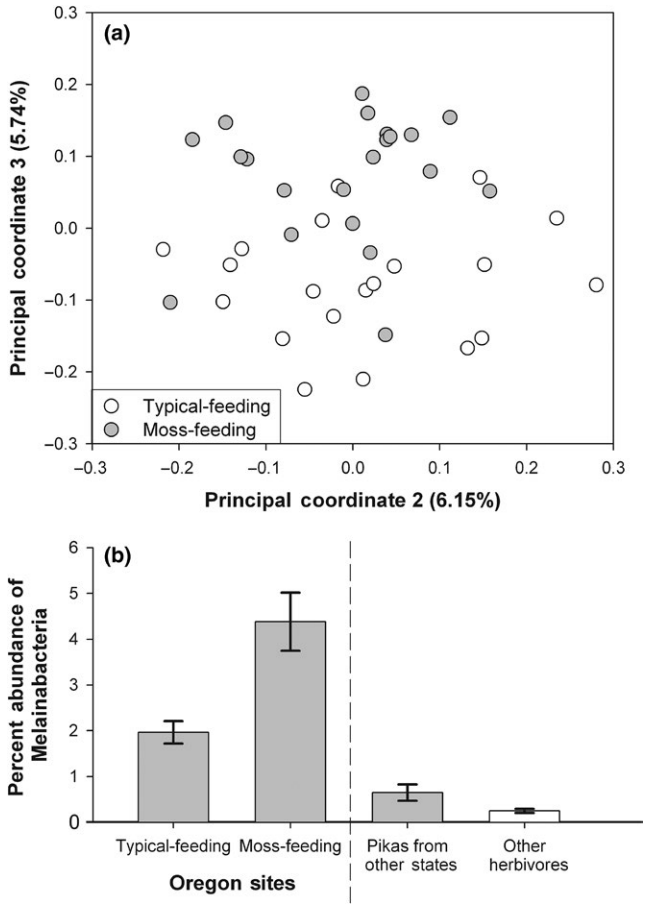

FIGURE 4 (a) Principal coordinate analysis of Oregon pika caecal pellet communities using Bray–Curtis distances. Points that cluster together share more similar microbial communities. (b) Relative abundance of Melainabacteria from Oregon pikas, pikas from other populations, and other mammalian herbivores (Di Rienzi et al., 2013)

(Gruninger, McAllister, & Forster, 2016). Members of *Coprococcus* isolated from rumen samples are able to degrade phenolic compounds, and these plant secondary compounds are common in the diets of pikas (Dearing, 1997; Patel, Jure, & Jones, 1981). The core microbiota also contained members of the genus *Oxalobacter* and the uncultivable family S24-7, both of which likely degrade oxalate, a common plant defensive chemical (Allison, Dawson, Mayberry, & Foss, 1985; Ormerod et al., 2016). Given that the plant material consumed by pikas is generally high in fibre and plant defensive chemicals, there may be strong selective pressure to maintain these fibre- and toxin-degrading microbes.

We were also interested in the microbial communities that might facilitate the ingestion of large amounts of moss by pikas in the Columbia River Gorge. Again, moss contains roughly double the fibre and significantly less nitrogen than typical food sources, and pikas in the Columbia River Gorge exhibit the highest moss intakes documented for any wild herbivore (Varner & Dearing, 2014). The most striking difference in microbial community structure for moss-feeding pikas was an enriched abundance of the candidate phylum Melainabacteria. This candidate phylum is closely related to Cyanobacteria, but has never been cultured in the laboratory (Di Rienzi et al., 2013). Relative abundances of Melainabacteria are higher in herbivorous mammals compared to those in other feeding groups, and in human populations that tend to consume high amounts of plant material (Di Rienzi et al., 2013). Genome reconstructions for Melainabacteria suggest that these bacteria are obligately fermentative, and thus may aid in the digestion of high-fibre plant material (Di Rienzi et al., 2013). Pikas from Oregon, especially those from sites where animals are known to feed on moss, exhibit the highest relative abundance of Melainabacteria for any mammal studied to date. This microbial phylum is present in the guts of herbivorous Asian pika species (Li, Li, Beasley et al., 2016), although at a lower abundance (<1% of the community). We hypothesize that these bacteria may be instrumental in allowing Oregon pikas to feed on high amounts of moss. It would be interesting to inventory the microbial communities of other herbivores that incorporate substantial amounts of moss into their diets, such as Svalbard reindeer (Bjrkvoll, Pedersen, Hytteborn, Jónsdóttir, & Langvatn, 2009) or brown lemmings (Batzli & Pitelka, 1983).

Our results also have implications for species conservation and management. While the American pika is not currently considered a threatened or endangered species, it is experiencing rapid population declines and extirpations due to climate change in parts of its range (Beever et al., 2016; Stewart et al., 2015). These declines have led some to call for the implementation of "assisted migration" to facilitate the colonization of future suitable habitats by pikas and conserve genetic diversity from threatened populations (Wilkening, Ray, Ramsay, & Klingler, 2015). While this proposal considers conservation of host genetic variation in such efforts, our study suggests that the microbiota of various source populations should also be considered. Should microbial variability of different host populations be maintained in conservation efforts? Will the gut microbial communities of source populations be compatible with the food material available in environments where pikas are introduced? Incorporating microbial ecology into the conservation strategies may be important for the preservation of this sensitive species and others.

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AUTHORS' CONTRIBUTIONS

K.D.K., J.V. and J.L.W. collected samples for analysis. K.D.K. conducted data analysis and wrote the manuscript. M.D.D. oversaw the study. All authors provided comments to improve the manuscript and approved the final version.

DATA ACCESSIBILITY

All data have been deposited on the Sequence Read Archive (SRA; https://www.ncbi.nlm.nih.gov/sra) under accession PRJNA343005.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

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