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PINWORMS ARE ASSOCIATED WITH TAXONOMIC BUT NOT FUNCTIONAL DIFFERENCES IN THE GUT MICROBIOME OF WHITE-THROATED WOODRATS (*NEOTOMA ALBIGULA*)

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KEY WORDS

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ABSTRACT

Vertebrates rely on their gut microbiome for digestion, and changes to gut microbial communities can impact host health. Past work, primarily in model organisms, has revealed that endoparasites disrupt the gut microbiome. Here, using wild-caught white-throated woodrats (*Neotoma albigula*), we tested whether naturally acquired parasite infections are associated with different microbiome structure and function. We surveyed wild *N. albigula* in eastern Utah for gastrointestinal parasites in the spring and fall of 2019, using traditional fecal float methods and testing a PCR-based approach to detect infection. We tested whether the host gut microbiome structure and function differed based on infection with the most prevalent parasite, the pinworm *Lamotheoxyuris ackerti*. In spring, infected and uninfected animals had significantly different microbiomes, but these differences were not detected in the fall. However, for both sampling periods, infection was associated with differences in particular microbial taxa determined by differential abundance analysis. As *N. albigula* rely on their microbiomes to digest both fiber and the plant defensive compound oxalate, we compared microbiome function by measuring dry matter digestibility and oxalate intake in infected and uninfected animals. Although we expected infected animals to have reduced fiber degradation and oxalate intake, we found no difference in microbiome function using these assays. This work suggests that parasite effects on the microbiome may be difficult to detect in complex natural systems, and more studies in wild organisms are warranted.

The vertebrate gut is a complex ecosystem with a dynamic interplay between host, microbes, and multicellular parasites. Vertebrates rely on their gut microbiome for digestive and immune functions (Hooper et al., 1998; Round and Mazmanian, 2009), and perturbations in the gastrointestinal tract can substantially alter the gut microbial community (Lewis et al., 2015; Iljazovic et al., 2021). Severe gut disturbances like antibiotics cause microbial depletion and dysbiosis (i.e., microbial imbalance) that can lead to illness (Wypych and Marsland, 2018; Cortés et al., 2019), while less targeted disturbances like gastrointestinal parasites cause complex, variable changes by both direct interactions with microbes and indirect interactions via the host immune system (Leung et al., 2018b; Rapin and Harris, 2018). Predicting how parasites impact the microbiome remains challenging, but studies of simplified systems and large populations have established patterns in parasite-induced microbial changes.

In the past decade, studies in captive animals and humans have demonstrated that parasites alter gut microbial communities (Zaiss et al., 2015; Leung et al., 2018b). In lab mice, *Trichuris muris* and *Heligmosomoides polygyrus* cause significant, consistent, and parasite-specific changes to gut microbiome structure (White et al., 2018; Rapin et al., 2020). In livestock, studies lack the replication of lab mouse systems but show that various gut parasites (e.g., *Haemonchus contortus* in sheep, *Trichuris suis* in pigs) alter host microbiomes across large populations (Li et al., 2012; El-Ashram et al., 2017). Similarly, anthelmintic therapy campaigns implemented with variable methods in humans have revealed both immediate and lasting effects of infection and clearance of pathogenic (e.g., *Trichuris trichiura*, *Necator americanus*) and more benign (e.g., *Enterobius vermicularis*) gut parasites on the gut microbiome (Yang et al., 2017; Rosa et al., 2018; Rubel et al., 2020). Combined, these data suggest that gastrointestinal parasites often alter microbial communities. However, the artificial environments and high infection intensities in these studies differ from conditions experienced by wild animals (Yakob et al., 2014).

Testing parasite-microbiome interactions in the wild is critical for understanding parasite impacts in the context of natural variation. In nature, infection intensity, gut microbial diversity, diet, and other factors vary on daily and seasonal timescales (Ren et al., 2017; Wu et al., 2017). Eliminating this variation increases the ability to detect microbiome changes, but environmental variability should be considered to accurately

understand parasitic impacts (Rosshart et al., 2017; Leung et al., 2018a). Furthermore, wild hosts are immunologically and physiologically distinct from laboratory animals, suggesting that wild animals and their microbes may also exhibit different responses to infection (Abolins et al., 2017; Bär et al., 2020). For example, in *Mus musculus*, *H. polygyrus* infection results in different microbial community changes in wild versus captive mice (Maurice et al., 2015; Kreisinger et al., 2015). There have been calls for broader sampling to understand how parasites shape the gut microbiome in wild hosts and if taxonomic shifts in the gut microbiota are functionally relevant (Dheilly et al., 2019; Trevelline et al., 2020).

Many wild microbiome studies focus exclusively on changes in community composition, but changes in composition do not always alter function (Barnes et al., 2020). Natural microbial communities show functional redundancy to overcome community shifts (Moya and Ferrer, 2016; Langille, 2018). Therefore, taxonomic analyses must be paired with functional assessments to understand how microbial changes impact hosts. In past parasite-microbiome studies, functional assessments have included predictive algorithms based on *16S* rDNA sequence identification (e.g., Phylogenetic Investigation of Communities by Reconstruction of Unobserved States 2.0 [PICRUSt2] Lee et al., 2014; Kreisinger et al., 2015; Douglas et al., 2020), gene catalogs from shotgun metagenomics (Rubel et al., 2020), and metabolomics (Houlden et al., 2015). These studies suggest that taxonomic differences translate to functional changes; however, predictive functional programs are often ineffective in wild systems (Djemiel et al., 2022). Direct measures of microbial digestion can bridge the gap between taxonomic and functional understanding. For example, fiber and dietary toxin degradation are direct measures of microbiome function relevant to mammalian herbivore health because gut bacteria degrade fiber and dietary toxins that mammals are unable to degrade (Dearing and Kohl, 2017). Wild herbivorous rodents and their parasites are excellent systems in which to examine parasite alteration of microbiome diversity and function due to their widespread distribution and ease of sampling.

To investigate natural parasite-microbiome interactions, we studied white-throated woodrats (*Neotoma albigula)* that feed heavily on *Opuntia* cactus (Miller et al., 2014). *Opuntia* spp. are rich in fiber (~50% dry matter) and oxalate (1.5–3.5% dry matter), which are both indigestible to mammals (Ramírez-Moreno et al., 2011; Kohl et al., 2015). In mammalian herbivores, fiber and oxalate are digested by symbiotic bacteria, and woodrats host a specialized gut microbiome that enables them to digest their fibrous diet and avoid oxalate toxicity (Justice and Smith, 1992; Lombard et al., 2014; Miller et al., 2014). As studies in laboratory rodents suggest that parasite infection can interfere with microbiome function, we hypothesized that *N. albigula* hosting intestinal parasites would host different bacterial communities and have reduced microbiome function compared to parasite-free animals. Specifically, we predicted that infected animals would have decreased microbial richness and significantly different community structures that would reduce fiber digestion and oxalate degradation. To test these predictions, we surveyed wild *N. albigula* for parasites, characterized their microbial communities, and measured their digestive function.

MATERIALS AND METHODS

Animal collection and husbandry

We collected adult (>95 g), non-pregnant *N. albigula* from the high-desert scrub habitat of Castle Valley, Utah (38°38′03.6″N, 109°22′02.1″W) in May 2019 (n = 15) and October 2019 (n = 24). Animals were collected in Sherman live traps and transported to the University of Utah's Bonderman Field Station at Rio Mesa to assay parasites, microbiota, and digestive function. Animals were housed in metabolic cages (Lab Products, Seaford, Delaware) so that all food and water intake, urine, and feces could be quantified for 4 days, and provisioned with ad libitum water and *Opuntia* cactus (CAC) from their capture site. We held animals for 4 days to gather 4 daily fecal samples for parasite screening, and to allow animals and their microbiomes to acclimate to the standardized cactus diet before taking fecal microbiome samples (Miller et al., 2017; Martínez-Mota et al., 2020). In May we fed animals whole cactus pads, and in October we fed animals powdered, dried cactus. Standardized housing and diet within sampling periods limited exposure to variable dietary and environmental microbes during digestive and microbiome assays. Each trial day we measured body mass and food intake to monitor animal health and collected feces for parasite screening.

We assayed digestive functions in October when animals were fed a powdered CAC diet. To prepare the powdered diet, we collected cactus, removed spines, finely chopped pads, and dried chopped material for 48 hr before grinding to a fine powder using an electric blade grinder. To measure oxalate intake, we transitioned animals to an oxalate-spiked (OX) diet for an additional 2 days immediately after the CAC diet, continuously housed in metabolic cages. As *N. albigula* tolerate up to 12% dietary oxalate (Miller et al., 2017), we expected differences in oxalate intake to be more detectable with concentrations closer to their maximum tolerance. To create the OX diet, we added sodium oxalate powder to CAC diet to reach an oxalate concentration of 8.5–10% oxalate by dry weight. Exact concentrations were calculated for each OX diet batch based on 1.5% average oxalate in Castle Valley *Opuntia* (Kohl et al., 2015), plus the dry weight of added oxalate.

Following diet trials, most animals were released at their capture site. Three animals from May were euthanized to collect adult parasites. Gut contents and tissue were separated by gut region, and contents were treated with near-boiling water and saved in 70% ethanol following methods from Justine et al. (2012). All animal capture and handling were approved by the Utah Department of Wildlife Resources (Scientific Collecting Permit 1COLL5914-2) and the University of Utah IACUC (Protocol 19-05006).

Parasitism

To detect parasite infections, we performed fecal flotations on 3 or 4 daily fecal samples from each animal. Each fecal sample consisted of approximately 20 fresh fecal pellets collected over a 24-hr period. For each sample, we homogenized 1.39 (± 0.41) g feces in 10 ml water, removed large plant fragments by washing through 1 mm mesh, suspended 2 ml of filtrate in 13 ml Sheather's sugar solution, centrifuged in a hanging bucket centrifuge (CL model, International Equipment Company, Needham Heights, Massachusetts) to concentrate eggs and oocysts, and scanned the

sample under ×100 total magnification following Dryden et al. (2005). We identified eggs and oocysts based on host, collection locality, and morphology as per Falcon-Ordaz et al. (2010) and Reduker and Duszynski (1985), respectively. We classified woodrats as infected if we detected multiple eggs or oocysts in at least 1 fecal float, and, for infected individuals, we calculated mean eggs per gram (EPG) and oocysts per gram (OPG) feces. We used linear models to test whether sex or body mass significantly differed based on sampling period or parasitism. We also calculated population-level infection prevalence and estimated the 95% Jeffrey's confidence interval (CI) for each parasite and sampling period. All averages are reported as mean ± standard deviation (SD), and all analyses were conducted in R v. 4.0.2 using the *prevalence* and *stats* packages (Devleesschauwer et al., 2015; R Core Team, 2020).

We amplified and sequenced parasite and CAC trial fecal samples (May, n = 15; October, n = 24) to confirm parasite identifications and test whether molecular methods provided more accurate diagnoses than fecal floats. To verify that protocols amplified target parasites, we used 2 adult nematodes collected from the cecum of 1 host and cecal material of another host shedding 1,554 coccidian OPG feces. We extracted DNA using DNeasy Blood and Tissue Kits (Qiagen, Hilden, Germany) for adult worms and PowerFecal DNA Kits (Qiagen) for cecal material, following manufacturer instructions. For both nematodes and coccidians, we amplified regions of *18S* rRNA. For nematodes, we targeted a ~900 bp region with nematode-specific primers Nem_18S_F (5′–CGCGAATRGCTCATTACAACAGC–3′) and Nem_18S_R (5′–GGGCGGTATCTGATCGCC–3′; Floyd et al., 2005). For coccidians, we targeted a ~1,600 bp region with coccidian-specific primers BT-F1 (5′–GGTTGATCCTGCCAGTAGT–3′) and hep1600R (5′–AAAGGGCAGGGACGTAATCGG–3′; Megía-Palma et al., 2016). PCRs contained 25 µl total volume, with 12.5 µl Q5 Hot Start High-Fidelity 2X Master Mix (New England Biolabs, Ipswich, Massachusetts), 0.5 pmol each forward and reverse primer, 1.5 pmol $MgCl_2$, and nuclease-free water. The amplification protocol for nematodes was 95 C for 5 min, then 35 cycles of 30 sec at 95 C, 1 min at 54 C, and 30 sec at 72 C, with a final extension step of 72 C for 7 min. For coccidians, we used the same protocol, but with an annealing step of 45 sec at 52 C. PCR products were cleaned using ExoSAP-IT Express (Thermo Fisher Scientific, Waltham, Massachusetts), DNA concentrations were quantified with a Qubit 4.0 Fluorometer (Thermo Fisher Scientific), and genomic DNA was sent to the University of Utah Sequencing Core for Sanger sequencing. Representative *18S* sequences were deposited to GenBank (OK142722–OK142724). After confirming that PCR protocols amplified nematode and coccidian samples, we screened feces from all animals (n = 39) for nematode and coccidian DNA. We extracted blank control samples and 0.25 g feces per animal using PowerFecal DNA Kits (Qiagen) and the above-described protocols, visualized PCR products on a gel, and then sequenced any sample that produced bands similar in size to target fragments.

Microbiome sequencing and bioinformatics

To characterize the gut microbiomes of animals fed CAC (May, n = 15; October n = 24) and OX (October, n = 23) diets, we collected feces at the end of each trial, froze fecal pellets at –80 C, and extracted DNA from feces as described above. Genomic DNA was sent to the DNA Service Facility at the University of Illinois Urbana-Champaign for PCR, library preparation (Naqib et al., 2018), and next-generation sequencing of the *16S* rRNA V4 hypervariable region using primers 515F (5′–GTGYCAGCMGCCGCGGTAA–3′) and 806R (5′–GGACTACNVGGGTWTCTAAT–3′; Caporaso et al., 2011). Samples were sequenced on an Illumina MiniSeq 2×150 bp platform (Illumina, San Diego, California) in June 2019 and January 2020. The ZymoBIOMICS Microbial Community Standard (Zymo Research, Irvine, California) included in both runs showed no significant difference between runs. All raw *16S* sequence data are available on the NCBI Sequence Read Archive (BioProject PRJNA757000, BioSample SAMN20931663).

We processed bacterial sequences in R, using *DADA2* 1.16.0 (Callahan et al., 2016) and *phyloseq* 1.32.0 (McMurdie and Holmes, 2013). After removing primers with *cutadapt* 2.5 (Martin, 2011), we filtered out sequences with Illumina quality scores lower than 20. We grouped sequences into amplicon sequence variants (ASVs; Callahan et al., 2017) at 100% sequence similarity and assigned taxonomy using the SILVA *16S* rRNA reference database v.138 (Quast et al., 2012), then constructed an approximately maximum-likelihood microbial phylogeny using FastTree 2.1.10 (Price et al., 2010). We removed sequences that were chimeric, represented by only 1 read, or identified as mitochondria or chloroplast. We also checked extraction and PCR blanks for evidence of contamination and found that these controls all contained fewer than 350 reads. As few woodrats hosted coccidians, we excluded animals with coccidian infection from microbial taxonomic and digestive functional analyses, restricting comparisons to individuals with the pinworm *Lamotheoxyuris ackerti* or no detected parasites. After filtering, May samples (n = 12) averaged 18,979 (± 2,016 SD) reads assigned to 524 ASVs, October CAC diet samples (n = 20) average 12,567 (± 5,729 SD) reads from 852 ASVs, and October OX diet samples (n = 19) averaged 10,526 (± 4,100 SD) from 715 ASVs. Filtered and trimmed sequence data and code are available on GitHub (https://github.com/mdoolin2014/albigula).

Microbiome community diversity and composition

To test whether bacterial diversity differed in infected and uninfected animals on the CAC diet, we calculated the number of observed ASVs (richness) and Shannon index (richness and evenness). We then tested whether these alpha diversity metrics differed between infected and uninfected animals using linear models and backward stepwise regression.

To test whether bacterial communities differed between infected and uninfected animals fed the CAC diet, we calculated 4 beta diversity metrics (Bray-Curtis, Jaccard, weighted UniFrac, and unweighted UniFrac). To examine how infection and sampling period influenced microbial communities, we first examined May and October samples together, testing the effect of sampling period and pinworm infection using the *adonis2* function to conduct a PERMANOVA. Communities significantly differed based on sampling period. Therefore, to account for potential seasonal differences in infection responses, we analyzed microbiome data based on pinworm infection separately for each period. We used the *phyloseq*, *microbiome* 1.10.0 (Lahti et al., 2017), and *vegan* 2.5-6 (Oksanen et al., 2019) packages for these

community analyses and confirmed model assumptions for PERMANOVA using the *betadisper* function. As all 4 distance metrics produced similar results, we present results from the Bray-Curtis metric.

We conducted differential abundance analysis to identify ASVs that differed in relative abundance between pinworm-infected and uninfected animals. Using fecal samples from animals on the CAC diet, we first looked for ASVs that were significantly enriched in either May or October. Then, within each sampling period, we looked for ASVs that were enriched in either infected or uninfected animals. We used the *DESeq2* 1.28.1 package for these analyses (Love et al., 2014), shrinking log2 fold changes using the *lfcShrink* function, with *ashr* 2.2-47 (Stephens, 2017) as the shrinkage estimator, and controlling the false discovery rate using the adjusted *P*-value cutoff of 0.05. For differentially abundant ASVs, we identified similar strains using *blastn* (NCBI, accessed 17 September 2021).

Finally, we tested whether parasitism was associated with significant microbiome differences when animals consumed a high oxalate diet. Comparing the fecal microbiomes after the CAC and OX diets, we first tested whether the OX diet altered bacterial alpha diversity, beta diversity, and bacterial abundances. Then for animals fed the OX diet, we tested how these metrics differed between infected and uninfected individuals, using the statistical methods described previously.

Digestive function

We tested whether pinworms were associated with reduced microbiome function by measuring dry matter digestibility and oxalate intake for October animals fed powdered CAC and OX diets. Dry matter digestibility (DMD) provides a metric of fiber digestion and is widely used to assay digestive efficiency in herbivores (Ramírez-Moreno et al., 2011). To calculate DMD, we measured 3 days of food intake and fecal output (dry weights) for individuals (n = 19) on the CAC diet, calculated DMD as 100 × (g ingested food − g feces)/g ingested food, and used the 3-day average DMD value for each animal (Dearing et al., 2000). After confirming suitability for parametric testing using the Shapiro-Wilk test ($P > 0.05$), we used a *t*-test to assess whether DMD differed based on pinworm infection.

Oxalate intake was used as a proxy for oxalate degradation. This is an effective proxy because woodrats reduce toxin intake when they reach their maximum tolerable dose, a result of toxin degradation being too slow compared to the rate of toxin intake (Miller et al., 2017). To calculate oxalate intake, we measured the amount of dry matter consumed by animals on the OX diet for 2 days. We then calculated oxalate intake based on dietary oxalate content, averaging intake over 2 days to calculate the average daily oxalate intake for each individual. After confirming that data were suitable for parametric tests, we used the *rstatix* 0.7.0 (Kassambara, 2021) and *stats* packages to perform an analysis of covariance, assessing whether oxalate intake varied based on pinworm infection while controlling for body mass.

RESULTS

Parasitism

Based on fecal floats, *N. albigula* (n = 39) from Castle Valley, UT hosted 2 parasite taxa: the pinworm *Lamotheoxyuris ackerti* and coccidian *Eimeria* sp. (Fig. 1). Pinworms (Fig. 1A) were the most prevalent parasite in both sampling periods, with higher prevalence in May (53.5%, 29.4–76.1% CI) than in October (20.8%, 8.4–39.8% CI; Fig. 1C). Although animals were infected with coccidians (*Eimeria* sp.; Fig. 1B), the prevalence was consistently low (May 13.3%, 2.9–36.3% CI; October 12.5%, 3.7–29.7% CI). Animals shedding nematode eggs averaged 12 ± 8.96 EPG feces (range 3–27), and animals shedding *Eimeria* sp. oocysts averaged 691 ± 1,009.43 OPG feces (range 15–2,349). The 3 necropsied woodrats had fecal floats with 0 EPG/0 OPG, 13 EPG/0 OPG, and 0 EPG/1,554 OPG feces, and yielded 4 (3M/1F), 2 (1M/1F), and 0 adult pinworms, respectively. Although host body mass differed between sexes (males > females) and sampling periods (October > May), body mass did not correlate with infection status ($R^2 = 0.298$, $F_{7,31} = 1.880$, $P = 0.107$).

Sequences for adult *L. ackerti* and *Eimeria* sp. aligned closely with rodent nematodes and coccidians, respectively. The *L. ackerti* sequences most closely matched (99.7% identity) several *Aspiculuris tetraptera* Nitzsch, 1,821 sequences (e.g., MH215350.1) from *Mus musculus*. The *Eimeria* sp. most closely matched (>96% identity) a sequence from *Eimeria scholtysecki* Ernst, Frydendall and Hammond 1967 (F324216.1) from an unspecified host and *Eimeria ferrisi* Levine & Ivens 1965 (KT360995.1, MH752036.1) from *M. musculus*.

Although we successfully sequenced adult nematodes and coccidian-infected cecal contents, PCR failed to detect parasite DNA in most woodrat feces. We amplified fecal samples from 39 hosts, including samples with nematodes (n = 15) and coccidians (n = 7) detected through fecal floats or necropsy. Although 10 samples produced bands at the approximate target sizes, 9 yielded off-target microbial sequences. The 1 successful sequence was from feces of the same coccidian-infected animal that yielded the coccidian sequence from cecal contents.

Microbial analyses

Microbial richness varied among individuals (139 ± 30 ASVs, range 82–194) and was not influenced by sampling period or infection status (ASV richness: $R^2 = 0.049$, $F_{3,28} = 0.479$, $P = 0.700$, Shannon diversity: $R^2 = 0.099$, $F_{3,28} = 1.028$, $P = 0.395$; Fig. 2A, B). Woodrats hosted microbial communities dominated by bacteria in the rodent-associated family Muribaculaceae (formerly S24-7, Lagkouvardos et al., 2019), as well as Lactobacillaceae and Lachnospiraceae (Fig. 2C).

Microbial diversity did not differ between sampling periods or infection status, but microbial community structure did (Fig. 3). When all samples were analyzed together, communities were significantly structured by sampling period (PERMANOVA, $R^2 = 0.142$, $P = 0.001$) but not parasitism ($R^2 = 0.049$, $P = 0.320$; Fig. 3A). Given the strong effects of sampling period, we analyzed May and October animals separately to test the effects of parasitism within each season (Fig. 3B, C). In May, microbiomes from animals with pinworms significantly differed from uninfected animals (PERMANOVA, $R^2 = 0.166$, $P = 0.006$). In October, infection had no impact on bacterial community composition (PERMANOVA, $R^2 = 0.039$, $P = 0.779$).

The relative abundance of individual ASVs also varied based on sampling period and infection status (Fig. 2D). When comparing the 2 sampling periods, we found 3 ASVs that were more abundant in May and 31 that were more abundant in

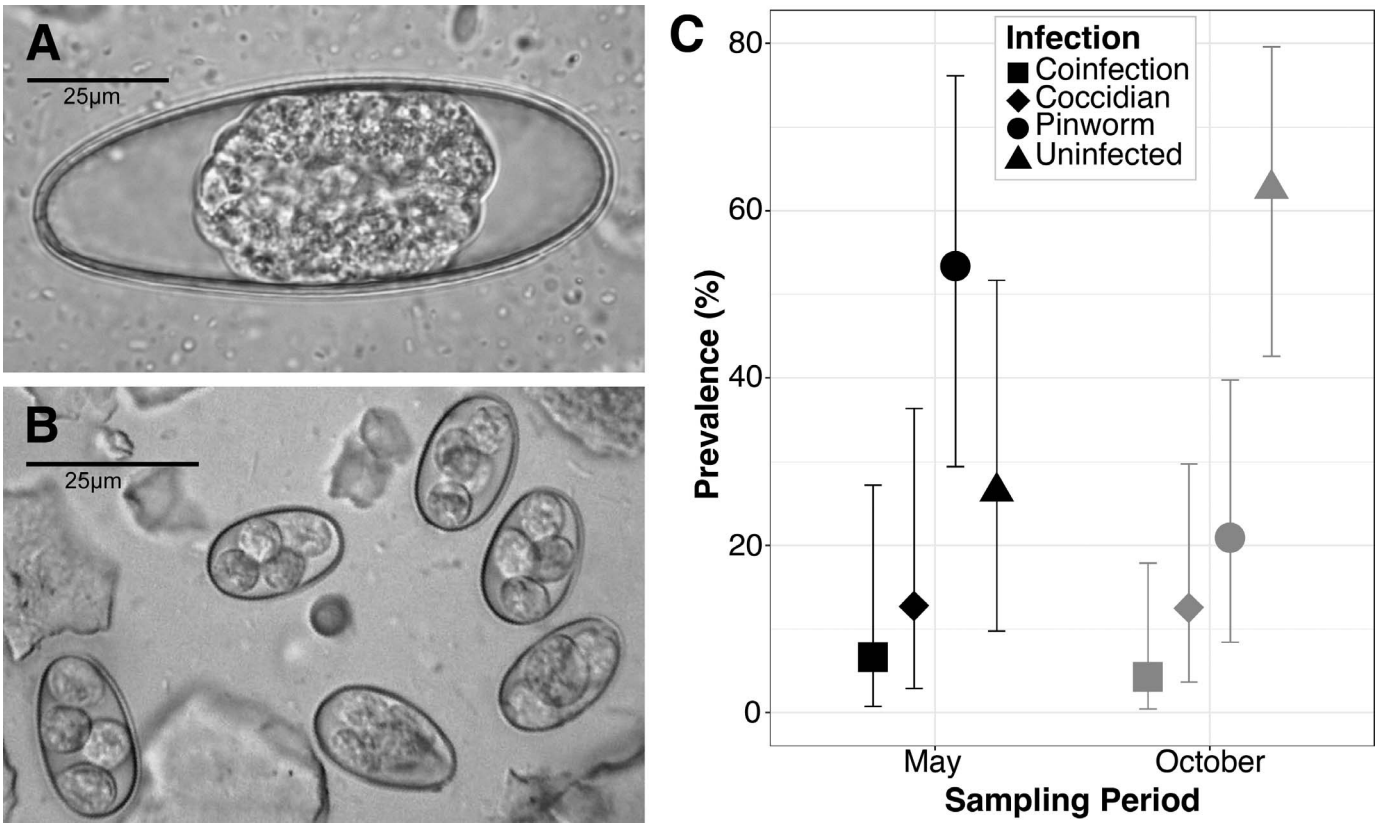

Figure 1. Transmission stages and prevalence of parasites in *Neotoma albigula*. Eggs of pinworm *Lamotheoxyuris ackerti* (**A**) and oocysts of *Eimeria* sp. (**B**) from woodrat feces. (**C**) Parasite prevalences during the May and October sampling periods. Error bars show 95% Jeffries confidence intervals of population-level prevalence.

October. In May, infected animals were enriched with a *Lactobacillus* sp. with 99.6% sequence similarity to uncultured *Lactobacillus* (MH078311.1) and *Lactobacillus gasseri* (MW131161.1), and an unidentified Prevotellaceae most similar to uncultured microbial sequences (MN474538.1, MH291713.1) detected from *Rattus* and an environmental sample, respectively. In October, uninfected animals were enriched with a strain of Muribaculaceae with >98% similarity to unidentified microbes (MF584015.1, KU339936.1) isolated from the Mongolian gerbil (*Meriones unguiculatus)* and soil, respectively, and a strain of Lachnospiraceae with >98% ID to *Robinsoniella* sp. (MH050588.1) from well water and an unidentified microbe (MF247670.1) from *Mus*.

The OX diet had no detectable effect on woodrat microbiomes, regardless of infection status (Suppl. Fig. S1). Although we predicted that increased oxalate consumption would alter gut microbiomes, switching from the CAC to OX diet did not alter alpha diversity (Fig. S1A), community composition (Fig. S1B, C), or individual ASV abundances (all analyses, $P > 0.05$). Although members of the microbial family Oxalobacteraceae were detected in more individuals after the OX spike than on CAC diet (11/19 animals compared to 1/20, respectively), they remained rare members of the community (<30 reads per woodrat).

Digestive function assessments

Pinworms had no detectable impact on digestive efficiency or oxalate tolerance. Infected animals had slightly, but not significantly, higher DMD (infected: 69 ± 2.0%; uninfected: 67 ± 3.0%; *t*-test, $P = 0.246$). Oxalate intake increased with body mass ($F_{1,16} = 8.172$, $P = 0.011$), but did not differ with infection status ($F_{1,16} = 2.004$, $P = 0.176$).

DISCUSSION

We investigated whether naturally acquired parasite infections were associated with taxonomic and functional differences in woodrat gut microbiomes. Pinworms, regarded as relatively benign (Mullink, 1970), were consistently the most common gut parasite in our study population. Pinworm infection was associated with different microbiome structure in May but not October, and we found no difference in microbial function between infected and uninfected animals measured in October. Our findings suggest that natural, low-intensity pinworm infections may alter gut microbiomes under some conditions, but other factors have greater impacts on microbial communities in complex habitats.

Neotoma albigula in Castle Valley hosted only 2 gut parasites, *Eimeria* sp. and *L. ackerti*, despite the rich endoparasite fauna of *Neotoma* spp. at other sites (Charles et al., 2012). Past surveys with similar sample sizes and methods found 7 parasite species in *Neotoma fuscipes* and *Neotoma macrotis* in California (Bechtel et al., 2015), and 7 parasite species in *Neotoma cinerea* in the Rocky Mountains (Miller and Schmidt, 1982). The depauperate gut parasite community in Castle Valley *N. albigula* could be due to a

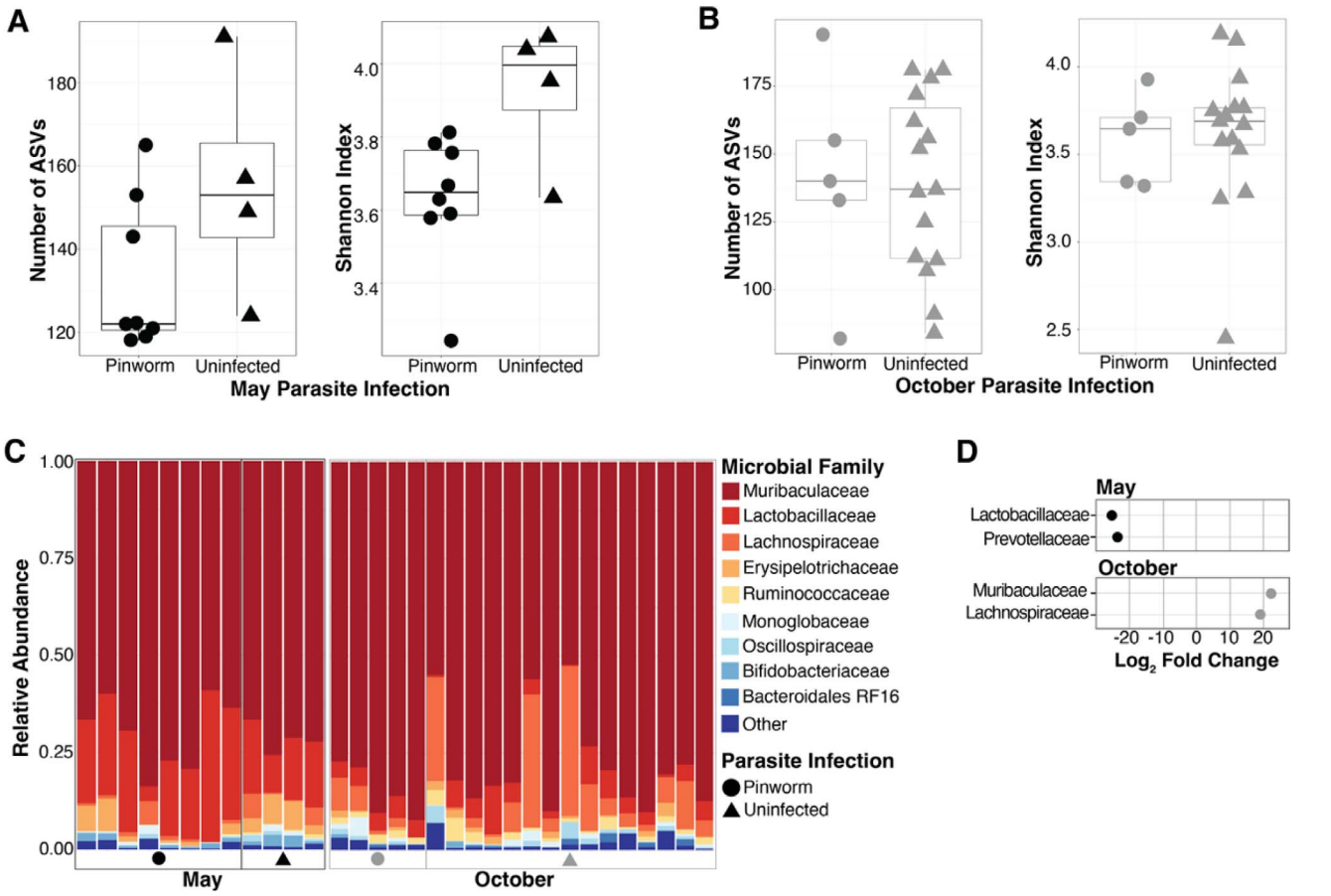

Figure 2. Alpha diversity and taxonomic composition of gut microbiota from uninfected and pinworm-infected *Neotoma albigula* after CAC diet trial. Amplicon Sequence Variant (ASV) richness and Shannon diversity of animals in May (**A**) and October (**B**) sampling periods. Each point represents 1 woodrat, and samples are divided in the boxplot based on sampling period and parasite infection. (**C**) Taxonomic composition of woodrat microbiota presented at the family level, divided by month and grouped based on parasite infection. Each vertical bar represents 1 woodrat. (**D**) DESeq2 differential abundance of ASVs with differential read counts based on parasite infection. Negative fold change indicates relative enrichment in pinworm-infected *N. albigula*. Each dot is 1 microbial taxon, labeled at the family level. Color version available online.

small host population, harsh environment, or our sampling methods. The site is at the northern limit of the *N. albigula* range in a high desert habitat surrounded by mountains that might limit immigration. Woodrat population sizes often fluctuate, and intensifying heat and drought at this site due to climate change may be exacerbating typical population lows (Calisher et al., 2005; National Weather Service, 2022). Under these circumstances, population densities may be below the threshold required to maintain some parasites (Arneberg et al., 1998). Additionally, increasingly hot and dry conditions could reduce intermediate host populations and decrease the survival of parasite stages outside of a host, perhaps explaining why we detected only parasites with fecal-oral transmission and highly resistant infective stages (Carlson et al., 2017). Alternatively, some parasite taxa may not have been found using our sampling and detection methods. Specifically, we may have missed rare parasites only present in young or pregnant individuals or those poorly detected by fecal floats.

Although necropsy is the most accurate method for detecting helminth infections, non-lethal methods are often needed in systems with limited or protected host populations. Fecal floats are a widely used, non-invasive method to detect gastrointestinal parasites but can underestimate richness and prevalence (Byrne et al., 2018). For example, we initially diagnosed a May animal as uninfected based on repeated fecal floats but recovered pinworms at necropsy. We attempted PCR-based detection to complement fecal floats and improve sensitivity (Aivelo and Medlar, 2018; Kwak et al., 2020), but our PCR approach failed to amplify parasite DNA from most infected hosts. Similar techniques have successfully characterized gut nematode communities in amphibians (Aivelo et al., 2018) and primates (Pafčo et al., 2019) and detected parasites in bird blood and excrement (Bourret et al., 2021). However, PCR-based approaches can fail due to unsuccessful lysis of resistant eggs during DNA extraction, PCR inhibitors or primer mismatch at DNA amplification, or no alignment of amplified sequences to existing genetic databases (Davey et al., 2021). Also, low-intensity infections are likely to be missed because DNA extraction often starts with a small subsample of fecal material (e.g., 0.25 g). Detection rates and precision can be increased by concentrating larger volumes of source material prior to DNA extraction or by using next-generation sequencing approaches (Scheifler et al., 2019). With these considerations, PCR-based detection may revolutionize non-lethal parasite surveys, but these approaches should comple-

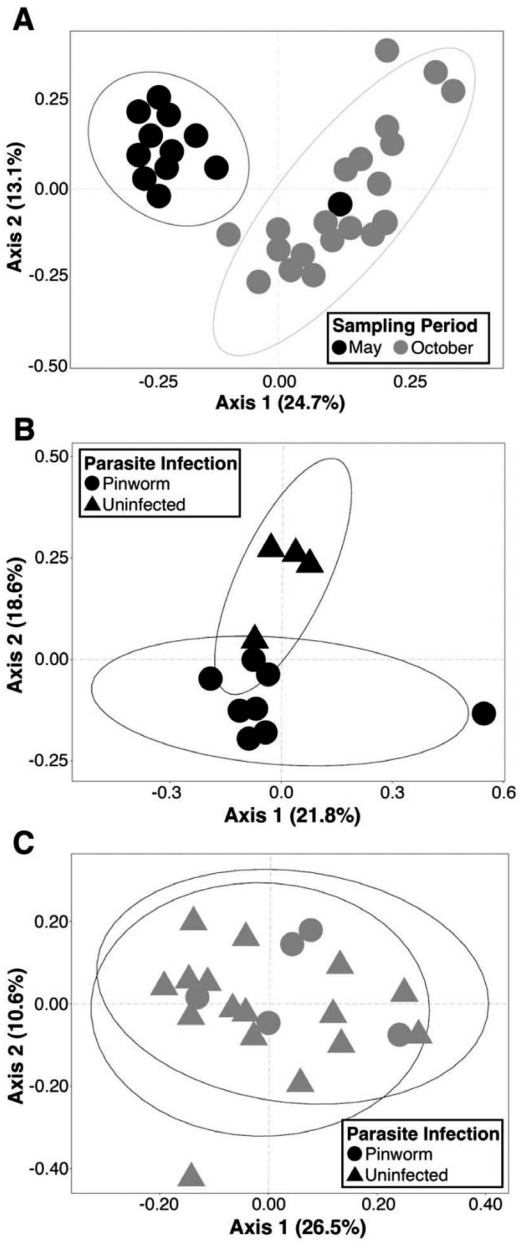

Figure 3. Principal coordinate analysis plots of Bray-Curtis ordination of *Neotoma albigula* gut microbiota after CAC diet trial. (**A**) May and October samples, where the sampling period explains microbial community composition but parasitism does not. (**B**) May 2019 samples, where microbial communities differ based on pinworm infection. (**C**) October 2019 sampling period, where microbial communities do not differ based on pinworm infection. (All ellipses are 90% confidence interval of ordinal space occupied by each group.)

ment traditional techniques (e.g., fecal floats, necropsy) until methods are standardized to ensure that parasite communities are accurately characterized.

In our survey, woodrats hosted complex gut microbial communities that substantially differed between sampling periods. The water content of the cactus diet in May versus October could have caused some microbiome differences, but providing ad libitum water in both sampling periods likely mitigated any large impact of whole versus powdered cactus. Strong seasonal differences in the microbiome are often seen in wild hosts due to changes in natural diets, reproductive condition, and animal behavior (Maurice et al., 2015; Youngblut et al., 2019). Like most wild hosts, *N. albigula* experiences seasonal changes in habitat and behavior that might alter their microbial communities. For example, differences between May and October animals could reflect changes in diet, as animals have access to more herbaceous plants in the spring. Additionally, differences could be due to changes in hormones, stress levels, and animal movement associated with springtime breeding in these otherwise solitary animals (Macêdo and Mares, 1988; Ezenwa et al., 2012). Critically, although we suspect that differences are due to season, sampling would need to be repeated across years to confirm that changes do not simply reflect microbial community drift between 2 sampling periods. Whether due to drift or season, microbiome differences between sampling periods overshadowed those attributable to pinworms.

Based on previous studies in humans, model organisms, and limited wild systems, we expected pinworms to alter wild *N. albigula* microbiomes. Pinworms impact the host immune system and feed on digesta in the cecum and large intestine (Adamson et al., 1992). Both of these effects could alter the host gut microbiome (Leung et al., 2018b). Since the host immune system governs the residential microbial community, inflammation and irritation at the infection site could cause the community differences we found in May 2020 (Yang et al., 2017). The host immune response could also have caused the differential abundance of particular microbial taxa found in each sampling period, or these differences could be due to pinworms directly feeding on microbes. It is unknown if pinworms selectively feed within the digesta, but selective feeding could significantly diminish preferred dietary microbes, especially in heavy infections. The effects of selective feeding are likely greatest at the infection site, and, as fecal samples contain microbiota from the entire gastrointestinal tract, we note that our non-lethal sampling may underestimate changes that are localized within a single gut region. Furthermore, in our study, low infection intensities likely limited both direct worm-microbe interactions and host immune responses (Sato et al., 1995). Therefore, the significant microbial community differences between infected and uninfected woodrats in May suggest that bacterial communities in these animals may have been more susceptible to parasite-induced perturbation, perhaps due to other environmental pressures (e.g., diet, reproduction). In contrast, animals in October have less diverse diets and no breeding interactions, potentially resulting in more stable microbial communities that are resilient to perturbation.

Consistent with the lack of microbiome compositional differences in October, we found no difference in microbial degradation of fiber and oxalate in October animals. We initially observed parasite-associated changes in May and predicted that we would find similar compositional differences in our second sampling period. The differences in parasite-associated microbiome changes between the 2 sampling periods underscores the complexity and unpredictability of wild systems. Functional changes may still occur when infection alters microbial community composition, but more studies are needed to understand when and how these changes occur.

In contrast to our predictions, the OX diet also did not result in significant microbiome change. We anticipated that increasing

dietary oxalate 5–6-fold for 2 days would cause oxalate-degrading bacteria to bloom in the gut, as has been observed in *N. albigula* dietary oxalate ramp-ups (Miller et al., 2016, 2017). The Oxalobacteraceae are particularly important oxalate degraders in the vertebrate gut that were never abundant in our study but were more prevalent after the OX diet than the CAC diet (Daniel et al., 2022). Their low abundance in the community may have been the result of their slow growth rate coupled with the short duration of the treatment (Jiang et al., 2011; Miller et al., 2017). They are also known to be rare in the woodrat gut microbiota (<1% of relative abundance; Miller et al., 2014), where the dominant family Muribaculaceae also contains facultative oxalate degraders (Lagkouvardos et al., 2019). Nonetheless, rare members of microbial communities can substantially contribute to overall function (Banerjee et al., 2018). The small increases in Oxalobacteraceae and facultative oxalate degradation by abundant microbes may explain the capacity of the woodrat microbiome to accommodate a large, abrupt increase in dietary oxalate without significant changes to microbiome structure.

Here we found that low-intensity pinworm infections in wild woodrats were associated with variable differences in gut microbiome structure. Results from this natural system differ from the strong parasite impacts documented in controlled laboratory settings, emphasizing the importance of characterizing these interactions in the context of natural variation. Environmental variability and low infection intensities may have modulated parasitic impacts in this system compared to studies of captive animals and humans. Future work in tractable wild systems could include experimental infection and deworming and other measures of digestive function (e.g., targeted metabolomics) to show causality and continue to improve functional understanding of microbiome changes. As every wild mammal hosts a microbiome and most host parasites, a more mechanistic perspective of host-parasite-microbiome interactions is critical to developing a complete understanding of how parasites impact host health.

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