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Rodents consuming the same toxic diet harbor a unique functional core microbiome

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Abstract

Gut microbiota are intrinsic to an herbivorous lifestyle, but very little is known about how plant secondary compounds (PSCs), which are often toxic, influence these symbiotic partners. Here we interrogated the possibility of unique functional core microbiomes in populations of two species of woodrat (*Neotoma lepida* and *bryanti*) that have independently converged to feed on the same toxic diet (creosote bush; *Larrea tridentata*) and compared them to populations that do not feed on creosote bush. Leveraging this natural experiment, we collected samples across a large geographic region in the U.S. desert southwest from 20 populations (~150 individuals) with differential ingestion of creosote bush and analyzed three gut regions (foregut, cecum, hindgut) using16S sequencing and shotgun metagenomics. In each gut region sampled, we found a distinctive set of microbes in individuals feeding on creosote bush that were more abundant than other ASVs, enriched in creosote feeding woodrats, and occurred more frequently than would be predicted by chance. Creosote core members were from microbial families e.g., Eggerthellaceae, known to metabolize plant secondary compounds and three of the identified core KEGG orthologs (4-hydroxybenzoate decarboxylase, benzoyl-CoA reductase subunit B, and 2-pyrone-4, 6-dicarboxylate lactonase) coded for enzymes that play important roles in metabolism of plant secondary compounds. The results support the hypothesis that the ingestion of creosote bush sculpts the microbiome across all major gut regions to select for functional characteristics associated with the degradation of the PSCs in this unique diet.

Introduction

The microbial communities of the mammalian gut are well known for their roles in defending against pathogens, training the immune system, and synthesizing nutrients [86]. These microbial communities can be highly variable among hosts due to differences in genetics, physiological status, and diet [113, 114]. Despite this variation, it is hypothesized that within a species, population, or dietary strategy, a subset of gut microbes or microbial functions persists, known as the "core microbiome".

These core microbes are proposed to be key ecological and functional members of the community, and recent studies reveal numerous insights into the microbial ecology found in an assortment of host species in a variety of environments [1, 58, 83]. For example, among domestic ruminants, a distinctive core microbiome occurs in the rumens of 32 host species [46]. Microbes belonging to the rumen core may represent taxa that are fundamental to this dietary strategy. However, microbial community structure does not always link with function as some functions are restricted to certain taxa while others are more widespread [103]. Thus, a host population may not share a similar taxonomic core of microbes, but may host microbes with shared functional capability, or a functional core microbiome. While many studies have investigated the presence of a shared, taxonomic core, few have extended the work to examine the functionality of the core.

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Stapleton *et al. Animal Microbiome*   (2024) 6:43

Page 2 of 17

In herbivores consuming a similar diet, core microbes may be involved in critical functions. Herbivores rely completely on their gut microbiota for fermentation of indigestible, complex plant carbohydrates, such as fiber and cellulose, into simple sugars usable by the host [63]. In addition, to deter herbivores, plants produce plant secondary compounds (PSCs), defensive toxins that can cause a wide range of negative physiological effects on the consumer [36]. It has long been hypothesized that herbivores house gut microbiota to aid in the degradation of PSCs [36] and recently, studies provide evidence for this microbial function in a multiplicity of herbivorous hosts [8, 10, 44, 53, 70, 71]. Plant secondary compounds also significantly influence the taxonomic structure of gut microbial communities [66, 105, 106]. Such a complex diet may select for a taxonomic core microbiome of organisms capable of fiber breakdown or interacting with PSCs. Alternatively, a lack of a discernable taxonomic core microbiome may mean that the ability to break down PSCs is more spread among various taxa, i.e., a functional core. While the presence of a core has been investigated across domestic, ruminant herbivores [46], there has been little investigation across different gut regions within a host, across different hosts consuming the same diet, or hosts in a natural setting.

The herbivore gut is highly specialized for ingestion of their complex diet, including adaptations for housing these communities of symbiotic microbes. Fiber degrading microbes reside in specialized, non-gastric stomach chambers in foregut fermenting animals such as ruminants [63] and in a more distal fermentation chamber, the cecum, for hindgut fermenting animals such as equids, rabbits, and rodents [63]. Some rodents, in addition to the cecum, have a sacculated foregut chamber proximal to the gastric stomach [20]. This chamber does not extensively ferment fiber [69] and houses microbes capable of metabolizing PSCs [69]. Little is known about the extent to which other species of herbivore host PSC degrading microbes and whether these microbes are similar across different host populations consuming the same diet [30].

To advance our understanding of how PSCs affect the gut microbiota of mammalian herbivores, we investigated the presence of a unique core microbiome in woodrat populations (*Neotoma* spp.) that have converged to feed on the same toxic diet, creosote bush (*Larrea tridentata*). These herbivorous rodents consume a variety of diets across their range [67, 114]. Some populations of both the desert woodrat (*Neotoma lepida*) and the Bryant's woodrat (*Neotoma bryanti*), species that diverged about 1.6 mya [92], independently converged to feed on creosote bush [80]. Creosote bush leaves are coated in a phenolic-rich resin composed of hundreds of PSCs such as phenolics, flavones, and saponins [3, 101]. Many of these compounds, such as nordihydroguaiaretic acid (NDGA) are toxic to mammals [31, 81, 98] and also antimicrobial [42, 74]. These PSCs not only strongly affect the diversity of the gut microbial community but may also select for microbes that use these compounds as substrates [105, 106]. Additionally, previous dietary intervention studies reveal that the gut microbiota play a critical role in facilitating ingestion of creosote bush in *N. lepida* [71]. Therefore, the PSCs in creosote bush may have selected for the same taxonomic or functional core microbiome in populations of *N. lepida* and *bryanti* that feed on creosote. To investigate the presence of a creosote-related core microbiome, we surveyed the microbial communities in 20 populations of both *N. lepida* and *bryanti* that consume creosote ("creosote feeders") and compared them to microbial communities from populations outside the natural range of creosote bush ("non-creosote feeders") to identify core microbes specific to a host diet rather than host species. Since microbial communities differ along the alimentary tract and previous work in this system was restricted to primarily microbes in the feces, we evaluated the three major communities in the gut, i.e., foregut, cecum, hindgut. We expected core microbes related to creosote feeding in the foregut as this structure has been documented to house microbes capable of degrading PSCs [67, 85]. Furthermore, we predicted that this chamber would harbor more gut microbiota capable of breaking down PSCs compared to the cecum, which should house primarily fiber degrading bacteria. Finally, because the most abundant PSC produced by creosote bush (NDGA) is a phenolic, we anticipated we would see microbial functions associated with the metabolism of xenobiotics and aromatic compounds in the functional core.

Experimental procedures
Sample collection
To examine the core microbiota of woodrats consuming a creosote diet, we collected 3–10 individuals from 20 populations across two woodrat species in 2017–2018 (n = 65 creosote feeders, n = 85 non-creosote feeders; Table S1; Figs. S1 and S2). Populations spanned the southwestern United States (California, Utah, and Nevada). We live-trapped animals using Sherman traps baited with oats; previous work has shown that this trapping method does not significantly affect the microbiome of woodrats [68]. For all individuals, a fecal sample was collected at the time of capture and animals were not released after sampling. Fecal samples were used as a representation of the hindgut microbial community because the microbiota in feces often resemble the hindgut of mammalian hosts [32, 33, 69]. Foregut and cecum contents were sampled from a subset of populations. These animals

Stapleton *et al. Animal Microbiome*   (2024) 6:43

Page 3 of 17

were dispatched after capture and immediately dissected (Table S1). Feces, foregut content, and cecum content were stored in liquid nitrogen in the field and then held at −80 °C until DNA extraction.

DNA extraction and amplicon sequencing

We isolated DNA from woodrat feces, foregut content, and cecum content using QIAamp PowerFecal DNA kits (Qiagen), following manufacturer protocols. Two negative controls were sequenced for each extraction kit. All DNA amplification, library preparation, and sequencing was conducted at the DNA Service Facility at the University of Illinois-Chicago [87]. To determine the gut microbial community of our sampled woodrats, we amplified the V4 hypervariable region of the 16S rRNA locus using the 515F and 806R primers following the Earth Microbiome Project suggested protocol [19]. To determine dietary content of each population, we amplified the P6 loop of the chloroplast *trnL* (UAA) intron using the *g* and *h* primers as previously validated and described [105, 106, 110]. Analysis was conducted at the population level as previous work has shown that using multiple samples can reduce bias from individual outliers and give a more accurate analysis of the diet [28, 105, 106]. In brief, starting PCR amplifications were performed in 10-µl reactions under the following conditions: 95 °C for 5 min, followed by 35 cycles of 95 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s. A second PCR amplification was performed wherein each sample well received a unique barcode from an Access Array Barcode Library for Illumina. Conditions for the second PCR were as follows: 95 °C for 5 min, followed by eight cycles of 95 °C for 30 s, 60 °C for 30 s and 72 °C for 30 s. Final libraries were pooled and size selected using AMPure XP cleanup (0.8×, v/v; Agencourt, Beckman-Coulter). All amplicon sequencing was conducted on an Illumina MiniSeq platform (2 × 150 bp paired-end reads).

16S rRNA sequence processing

All 16S sequences were processed in QIIME2 version 2021.8 [13]. Primers were removed using Cutadapt [82] and resulting sequences were filtered for quality control in QIIME2. Sequences were grouped into amplicon sequence variants (ASVs) using DADA2 [18], resulting in 5,508 unique ASVs that were assigned to taxonomy using the Silva database release 138.1 [94]. We removed identified chimeras, sequences that appeared in fewer than four samples, sequences that appeared less than ten times total across all samples, and sequences that were identified as chloroplast or mitochondria. After filtering, samples contained 6,884,401 total reads with an average of 27,985 reads per sample. To control for differential sequencing depth, samples were rarefied to a sequencing depth of 4499 reads per sample (the lowest coverage in any one sample that contained sufficient reads). Rarefaction resulted in the removal of four samples. Identification of core microbes was done using unrarefied data, as rarefaction can skew the estimation of core microbes [88, 95]. All other analyses were performed on rarefied data.

trnL sequence processing

To determine the diets of animals, we used *trnL* plant metabarcoding on fecal samples. Plant sequences were processed using QIIME2 version 2021.8 as previously described and validated [105, 106]. In brief, we retained high-quality sequences by setting minimum sequence length after trimming to 20 bp, increasing minimum acceptable PHRED score to 20, and reducing minimum overlap to 10 bp, all other parameters were left at default settings. Sequences were assigned to operational taxonomic units (OTUs) at the 100% identity level using denovo clustering. Chimeras were removed and, based on the contents of sequencing blanks, we removed OTUs represented by fewer than twenty reads per sample and any OTUs that appeared in less than five total samples. Samples with >1000 total reads before filtering were removed from this analysis (3 samples). Taxonomy was assigned to OTUs using the Scikit-learn classifier in qiime2 version 2021.8, trained on a custom reference database. To create our database, we used a custom python script to download chloroplast sequences and taxonomies from the NCBI nucleotide database, generate a FASTA file containing each sequence and its reverse complement, and trim the FASTA file using Cutadapt 2.10 with the 5′ and 3′ adapters set to the *trnL* g and h primers, as previously described [114]. Sequences were not included in the reference database if they contained ambiguous nucleotides, mismatches in either primer, more than three mismatches overall, an amplicon length outside the range of 8–175 bp, contained a taxonomic classification lacking "Viridiplantae", or if the sequence was flagged as "environmental_samples". Sequences with taxonomy that did not resolve to at least the family level were considered unclassified (<4% of sequences). After examining the content of our negative controls and based on previous work [105, 106], families that represented less than 1% of each population's total relative abundance were removed. Populations were considered 'creosote feeders' if creosote reads were present in diet samples after this filtering step and if creosote occurs in the region the population was sampled from Tables S3 and S4.

Identification of core members

To determine the creosote-feeding core microbiome, we used a two-step process. First, we identified the core

Stapleton *et al. Animal Microbiome*   (2024) 6:43

Page 4 of 17

ASVs across all woodrat samples as those that were present in ≥50% of *N. lepida* and *N. bryanti* samples [78, 99, 113]. This threshold was chosen based on precedence from other studies that suggest using a more stringent threshold generates diversity scores that correlate poorly with unfiltered data and reduces the ability to compare core microbiomes across studies. Also, because we sampled across many geographically distinct populations, which is known to significantly influence the structure of the microbiome [1, 39, 40, 45, 55, 99, 108, 114]. Then, we removed all core woodrat microbes that were not unique to creosote feeding woodrats to yield the creosote core. Using this two-step process, we defined core ASVs for the cecum, foregut, and hindgut of *N. lepida* and *N. bryanti* in creosote feeding populations and in non-creosote feeding populations. We considered ASVs part of the 'creosote-feeding core microbiome' for each sampled gut region if they met the ≥50% threshold for the general core in both *N. lepida* and *N. bryanti* samples and were not considered core in non-creosote feeding populations. Microbes meeting these thresholds are designated as 'core' or 'creosote-feeding core' hereafter. In addition, because we had more hindgut samples than foregut and cecum, we evaluated the effect of sample size on estimates of core microbes, by restricting the hindgut dataset to only samples that had a matching foregut and cecum sample, and comparing the results from this subset to the results from all hindgut samples.

Statistical analysis

Alpha-diversity of the microbiome was measured using Shannon's index and Observed ASVs, these values were compared across the foregut, cecum, and hindgut using Kruskal–Wallis tests and between creosote feeders and non-creosote feeders using Mann–Whitney U tests implemented in R. We measured beta-diversity using Bray–Curtis distances (community structure) and Jaccard distances (community membership). Differences in beta-diversity were compared using permutational multivariate analysis of variance (PERMANOVAs) implemented using the vegan package in R with diet type, species, and population as factors. To determine whether core ASVs were under host selection, we fit the prokaryotic neutral model to all ASVs found in each gut region following methods described in [17]. Using this model, all ASVs were classified as either over-represented, neutral, or under-represented. In addition, we applied the neutral model to each population to determine whether there was greater selection on creosote populations than non-creosote populations. Finally, we estimated differentially abundant ASVs using DESeq2 [79]. We used the package ashr to shrink log2fold changes generated by DESeq2, changes in relative abundance were considered significant if the FDR corrected *p*-value was < 0.01 and the log fold change was ≥1.5.

Metagenomic analysis

We performed metagenomic sequencing on 45 fecal samples from 9 populations (n = 3 per species, per population; Table S3). We extracted DNA as previously described, library preparation, amplification, and final sequencing were completed at the DNA Service Facility at the University of Illinois-Chicago. Library prep was performed using the Swift 2S Turbo DNA Library Kit with enzymatic fragmentation (catalog 44,024 Swift Biosciences Ann Arbor, MI) followed by PCR performed according to the manufacturer protocol. Final libraries were size-selected, pooled, and sequenced on an Illumina NovaSeq 6000 with 2 × 150 bp sequencing and a 1% phiX spike-in. Metagenomic sequencing resulted in a total of 452,316,506 reads with an average of 10,051,478 reads per sample (S.D. 1,719,434).

Previous research has shown that read-based and assembly-based methods can produce different results [111], therefore, we characterized the functional profile of the gut microbiota using both unassembled and assembled reads. For the unassembled reads, we used MEGAN6 to conduct an analysis of gene function on forward reads [50]. Adaptors were trimmed from all sequences using FastP [22]. We removed host reads from sequences by mapping the reads to all of the following host genomes: *Peromyscus leucopus*, *P. maniculatus*, *P. nasutus*, and *Neotoma lepida*. We ran DIAMOND (v. 2.0.9) [16] to blast the remaining reads against the UniRef100 database [109] with an e-value cut-off of 0.001. These host-filtered, annotated forward reads were uploaded into MEGAN6, community edition and classified to KEGG Orthologs (KOs) using the Kyoto Encyclopedia of Genes and Genomes (KEGG) database [62]. Genes were classified as belonging to the creosote core microbiome as previously described for the taxonomic core. We used DESeq2 to investigate genes that were differentially abundant between creosote and non-creosote feeders as previously described.

In addition, to identify bacteria associated with creosote feeding, we assembled metagenomic sequences into metagenome assembled genomes (MAGs). We used Metaspades (SPAdes v. 3.15.3) on a large memory node to co-assemble all 45 samples resulting in 16,336,611 total contigs. Then, we used MetaBat2 to bin contigs with default parameters, resulting in 649 total bins. We measured MAG completeness and contamination using checkM (v. 1.1.3) and dereplicated the MAGs to 99% ANI using dRep [90]. All unique MAGs identified as > 50% complete and with < 10% contamination were kept for further analysis, resulting in 271 total MAGs identified

Stapleton *et al. Animal Microbiome*   (2024) 6:43

Page 5 of 17

across all samples. We classified MAGs as part of the 'core creosote-feeding microbiome' using the methods described above for ASVs (i.e., creosote-feeding core). We assigned taxonomy to the MAGs using the Genome Taxonomy Database (GTDB) and GTDB-Tk [21]. The functional capability of core MAGs was determined by identifying KOs in each sample. First, coding regions were identified using Prodigal and then extracted using GffRead [93]. To reduce gene redundancy, we clustered the resulting genes at 95% sequence similarity using CD-HIT [37]. Non-redundant genes were kept and blasted against the UniRef100 database using DIAMOND for functional annotation. We then generated gene abundance profiles by mapping reads from each sample to non-redundant genes using BWA [77]. We investigated the presence of differentially abundant genes in creosote and non-creosote feeders using DESeq2, which has been validated for use with metagenomes [57].

Results

Community composition across and within gut regions

For all woodrat populations, gut microbial diversity varied across the foregut, cecum, and hindgut. Alpha-diversity metrics were significantly different across the three gut communities (Kruskal–Wallis, Shannon index $H(2) = 84$, $p < 0.001$; Observed ASVs $H(2) = 68$, $p < 0.001$; Fig. S3). The cecum proved highly diverse, with the highest alpha-diversity for both the Shannon index and observed ASVs while the foregut was the least diverse (Table 1). Differences in gut microbial diversity between creosote feeders and non-creosote feeders varied by gut region (Table S4). In the cecum and the hindgut, non-creosote feeders showed increased alpha-diversity compared to creosote feeders when measuring the Shannon index and observed ASVs, respectively (Cecum, Mann–Whitney U, Shannon index $U = 160$, $p < 0.01$; Hindgut, Mann–Whitney U, Observed ASVs $U = 1878$, $p < 0.02$). There was no difference in alpha-diversity between the gut microbial communities of creosote and non-creosote feeders in the foregut.

The microbial community of the three gut regions significantly differed in both community membership (PERMANOVA, Jaccard, pseudo-F = 4.6, $R^2 = 0.04$, $p < 0.001$) and community structure (PERMANOVA, Bray–Curtis,

Table 1 Average alpha-diversity metrics (Shannon's Index and Observed ASVs) found in each gut region

	Avg. Shannon's Index	SE	Avg. observed ASVs	SE
Foregut	4.3	±0.87	307	±142
Cecum	5.4	±0.34	499	±82
Hindgut	5.0	±0.33	358	±93

pseudo-F = 7.7, $R^2 = 0.06$ $p < 0.001$). The variable with the most explanatory power was the most abundant plant family in host diet rather than gut region (PERMANOVA, Jaccard, diet $R^2 = 0.14$, $p < 0.001$; Bray–Curtis, diet $R^2 = 0.20$, $p < 0.001$). Within each gut region, the microbial communities of creosote-feeding populations significantly differed from populations that do not feed on creosote (PERMANOVA, Jaccard and Bray–Curtis, $p < 0.001$ for all gut regions; Fig. 1) with levels of dietary creosote explaining 7% of the variation (PERMANOVA, Jaccard and Bray–Curtis, $R^2 < 0.07$, $p < 0.001$ for all gut regions; Table S5).

Creosote-feeding core microbiome membership

The cecum, foregut, and hindgut, predominantly harbored distinct creosote-feeding core microbiomes. With respect to the core microbiome unique to creosote feeders, hereafter 'core', 25 ASVs were identified as being core in more than one gut region, while two ASVs, both in the family *Lactobacillaceae* were core microbes across all three regions (Fig. 2A). The majority of core ASVs shared between two gut regions belonged to *Muribaculaceae* (Table S6). The cecum harbored the largest overall core microbiome of the gut regions. Of the 2973 total identified ASVs in the cecum, 164 were classified as core (Fig. 2C) represented by 18 microbial families. The families containing the most core ASVs in the cecum were *Lachnospiraceae* (64 ASVs), *Oscillospiraceae* (26 ASVs), and *Muribaculaceae* (29 ASVs).

The foregut had the smallest core of the three gut regions (Fig. 2B). The foregut harbored a total of 2207 identified ASVs, 36 of which were identified as core. Foregut core ASVs were represented by 9 families with *Muribaculaceae* (12 ASVs), *Lachnospiraceae* (6 ASVs), *Desulfovibrionaceae* (5 ASVs), and *Eggerthellaceae* (3 ASVs) containing the most. Finally, hindgut samples contained a total 3781 identified ASVs with 40 ASVs classified as core, belonging to 10 families (Fig. 2D). *Muribaculaceae* and *Oscillospiraceae* also contained the most core hindgut ASVs, followed by the family *Lachnospiraceae* (15, 6, and 5 ASVs respectively).

The sample size of the hindgut dataset affected the size of the core microbiota. When we restricted our hindgut samples to match the animals included in the cecum and foregut samples, the number of microbes identified as core increased. For the smaller hindgut dataset, we saw 2,627 ASVs, 66 of which were classified as core. Core microbes were represented by 13 different families, with the majority of ASVs belonging to the *Muribaculaceae, Lachnospiraceae,* and *Oscillospiraceae* (25, 12, and 9 ASVs, respectively). None of the identified core members belonged to the *Butyricoccaceae*, despite that family being present for core members in
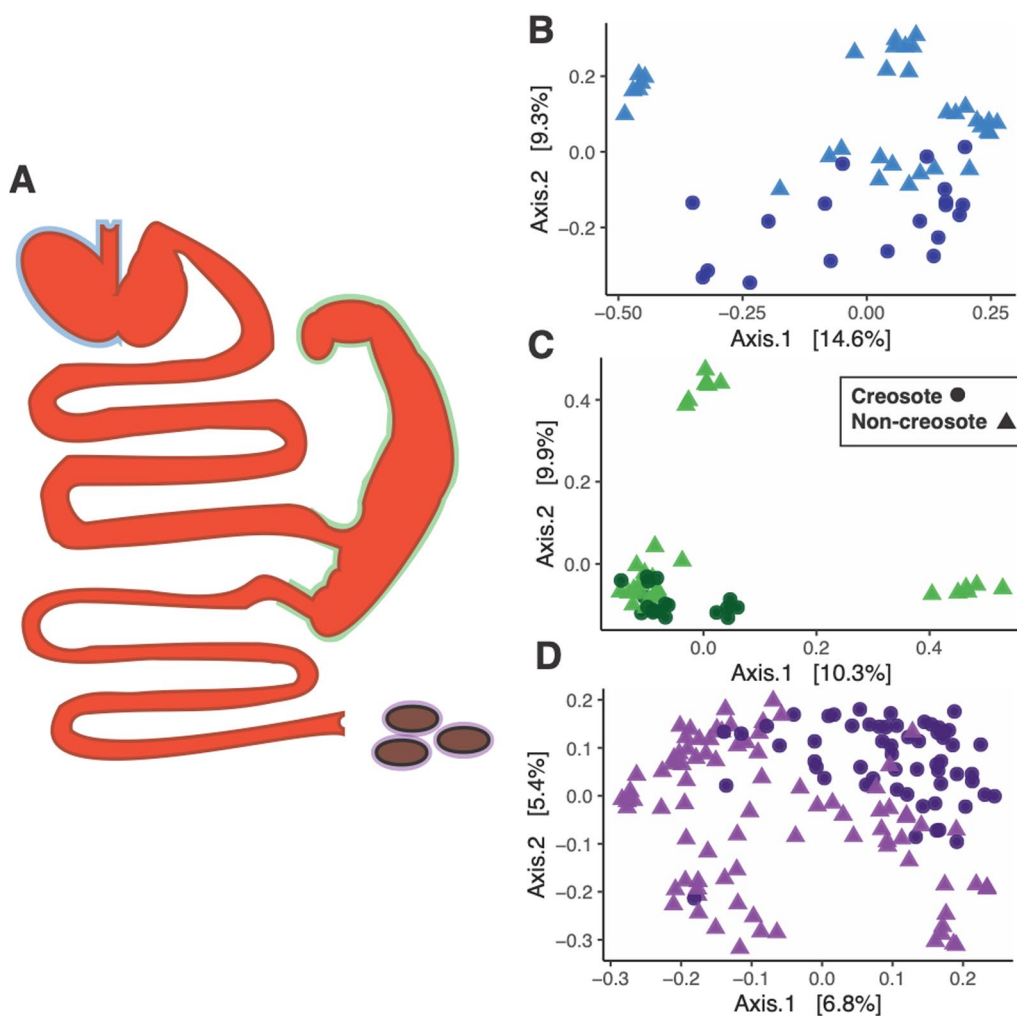
Fig. 1 Gut microbial community structure and membership between creosote and non-creosote feeding woodrat populations. **A** Diagram of the gastrointestinal tract of woodrats, highlighted segments represent sampled gut regions: the foregut (blue), cecum (green), and hindgut (purple). Principle coordinate analysis of Bray–Curtis distances between creosote and non-creosote feeders for the foregut (**B**), cecum (**C**), and hindgut (**D**); there were significant differences between creosote and non-creosote feeders across all gut regions (statistics reported in the text)

the full hindgut core dataset. Notably, though the core microbiota increased compared to the larger hindgut dataset, the cecum still harbored a larger core microbiome.

Core ASVs did not comprise the majority of the microbiome, but were more abundant than other ASVs. The relative abundance of core ASVs was significantly higher than non-core ASVs within each gut region (Kruskal–Wallis, $p < 0.001$, cecum, foregut, and hindgut). Within each gut region, the average relative abundance of any one core ASVs never exceeded 1.5%. In the cecum, the most abundant core ASV belonged to the family *Lachnospiraceae,* and had a total relative abundance across all creosote-feeding samples of

0.07%. Collectively, cecum core microbes comprised a total relative abundance of 19.1% while core foregut ASVs comprised 15.7% of the total. The most abundant core ASV in the foregut had the highest relative abundance of any core ASV across the gut regions with a total relative abundance of 6.1% (family *Lactobacillaceae*). The most abundant core ASV in the hindgut also belonged to the family *Lactobacillaceae,* and had a total relative abundance in all creosote-feeding samples of 1.1%. The cumulative relative abundance of all core ASVs in the hindgut was 10.4% of the microbiome. Though no core ASVs comprised the majority of the microbiome in any gut region, no single ASV outside the core had a higher relative abundance than 7.6% (foregut, non-core ASV).
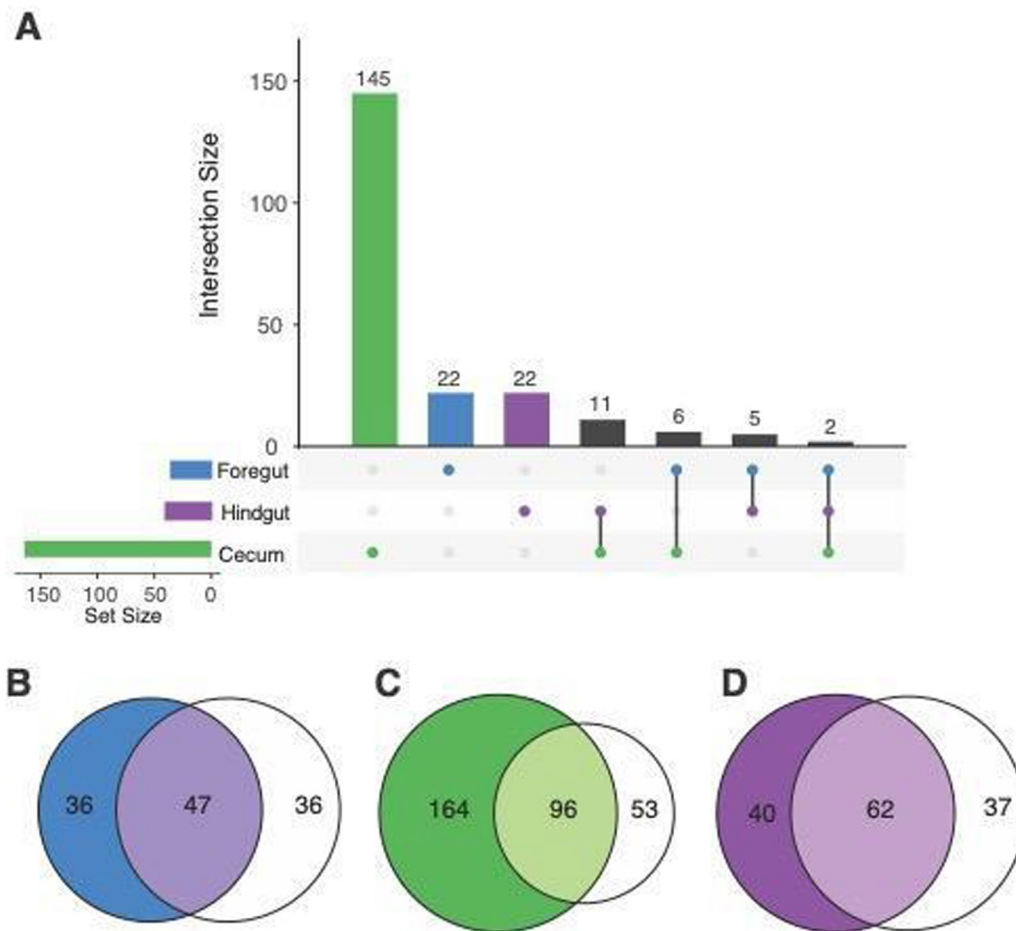
Stapleton *et al. Animal Microbiome*   (2024) 6:43

Page 7 of 17

Fig. 2 Gut regions harbored distinct core microbiotas. **A** UpSet diagrams of core microbes shared between all gut regions. Few core microbes were shared across gut regions. Euler diagrams of core microbes (number of ASVs) that were unique to creosote feeders (left), unique to non-creosote feeders (right), and shared by all populations (center) in the foregut (**B**), cecum (**C**), and hindgut (**D**)

Taxonomic differences in creosote feeding populations

Core ASVs were significantly enriched in the cecum, foregut, and hindgut of creosote-feeding populations compared to non-creosote feeding populations. Using DESeq2, within the cecum, we found 111 ASVs belonging to 13 microbial families significantly enriched in creosote feeding animals compared to non-creosote feeding animals; 36 of these ASVs were core (Fig. 3A). Enriched, core ASVs were represented by 9 microbial families (Table S7). Amplicon sequence variants with the largest logfold changes compared to non-creosote feeders belonged to the families *Muribaculaceae* and *Lachnospiraceae* (Table S7). Forty-eight ASVs were significantly enriched in the foregut of creosote feeding animals, 16 of which were core (DESeq2; Fig. 3A). Significantly enriched core ASVs in the foregut belonged to the families *Eggerthellaceae*, *Lactobacillaceae*, *Lachnospiraceae*, and *Pasteurellaceae* (Table S8). The hindgut of creosote feeders contained the highest number of significantly enriched microbes compared to non-creosote feeders

(See figure on next page.)
Fig. 3 Core microbes enriched and selected for in the foregut, cecum, and hindgut of creosote feeders. **A** Log2 fold enrichment of core microbes in creosote feeders compared to non-creosote feeders. **B** Deviance of core ASVs from neutral model fit. Overrepresented ASVs appear above the line and underrepresented ASVs beneath the line. Points are colored by gut region. **C** Fit of the prokaryotic neutral model to all ASVs in the foregut (blue outline), cecum (green outline), and hindgut (purple outline). Closed circles represent members of the core microbiome, open circles represent non-core ASVs. ASVs are classified as overrepresented (yellow), underrepresented (red), or neutral (gray). The solid line represents the predicted frequency of occurrence and the dashed line is 95% confidence intervals
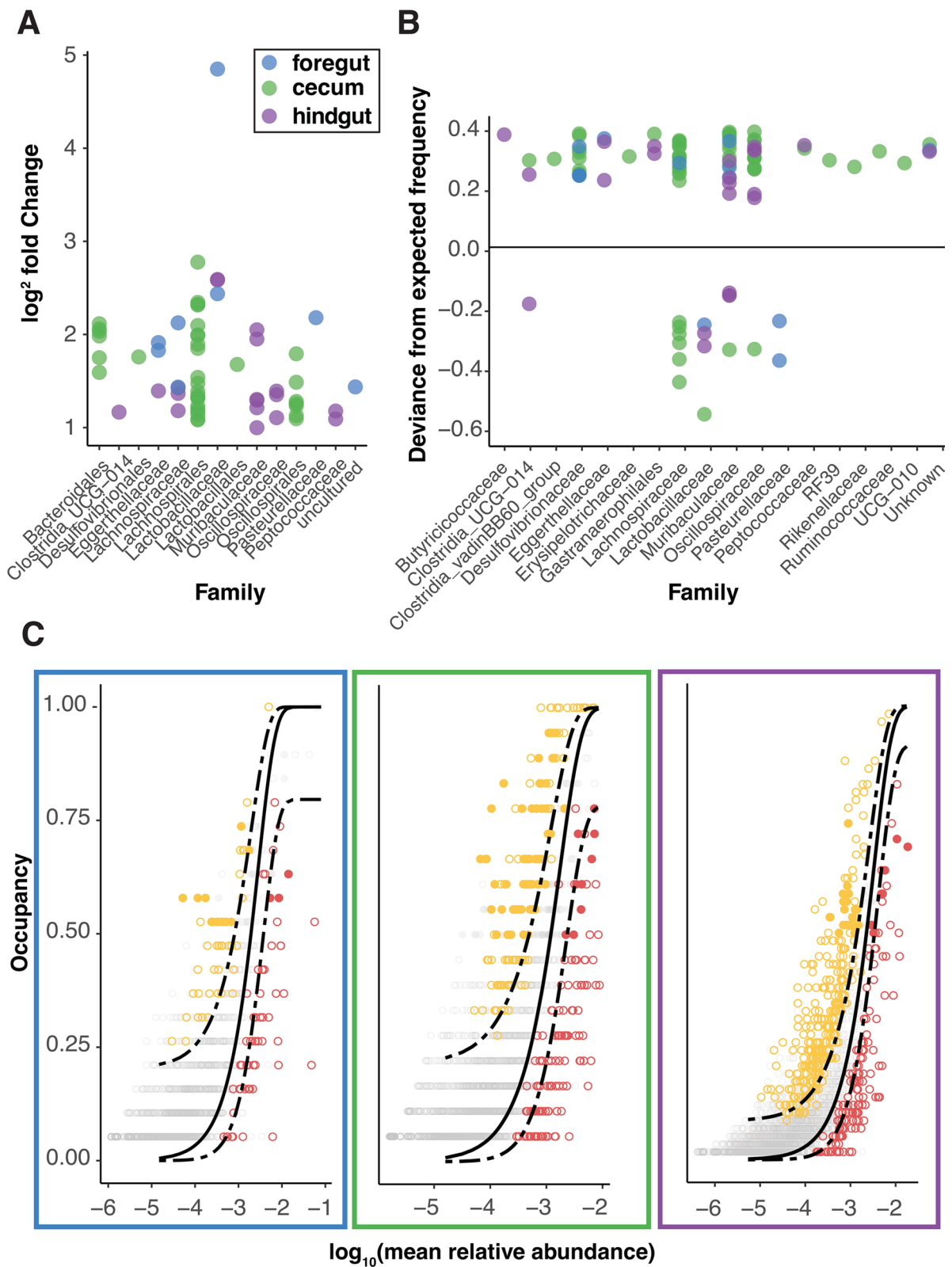
Fig. 3 (See legend on previous page.)

with 151 enriched ASVs, 18 of which were classified as core (DESeq2; Fig. 3A). These enriched core microbes were represented by 7 microbial families; the largest log-fold changes were observed in ASVs belonging to the *Muribaculaceae* and *Lactobacillaceae* (Table S9).

Neutral selection of ASVs

To determine whether core ASVs were under selection, we assessed the fit of the prokaryotic neutral model to occupancy and abundance distributions of all ASVs in each gut region. Deviations from this model (i.e., reduced model fit) are indicative of non-neutral processes, such as selection from the host or host diet [17]. Model fit was greatest for all ASVs in the hindgut ($R^2 = 0.46$); however, when we restricted the dataset to only include hindgut samples from hosts that had matching cecum and foregut samples, the model fit decreased ($R^2 = 0.27$) and was more similar to the other gut regions (cecum $R^2 = 0.30$; foregut $R^2 = 0.26$). Additionally, there was no significant difference in prokaryotic neutral model fit on all ASVs between creosote and non-creosote populations (student's *t*-test, $t(13) = -0.31$, $p = 0.78$) nor was there a relationship between the amount of creosote in each population's diet and model fit (linear regression, $R^2 = 0.24$, $p = 0.12$). Amongst the ASVs identified as under selection, many were core microbes from each of the different gut regions. Fewer core microbes selected against, or underrepresented than under selection (Table 2). Across all gut regions, there was underrepresentation of core ASVs belonging to the *Lactobacillaceae, Muribaculaceae, Clostridia_UCG-014, Pasteurellaceae, Lachnospiraceae,* and *Oscillospiraceae* and overrepresentation of core ASVs belonging to many families found in the core (Fig. 3B, C).

Characterizing the functional core microbiome

To characterize the potential functional core microbiome of creosote feeding woodrats, we compared the gene content of the gut microbial communities of creosote and non-creosote feeding woodrats using KOs. Using the unassembled reads, we investigated the abundance of high-level functional pathways and found that most functions were unclassified (46%) with the next most

Table 2 Proportion of core microbes identified as being selected for or selected against by the prokaryotic neutral model

	Core selected for (%)	Core selected against (%)
Foregut	30.50	8.30
Cecum	39.02	5.49
Hindgut	47.50	15.00

abundant pathways being functions associated with metabolism (Fig. 4A; Table S10). We also investigated whether there existed a functional core in creosote feeders using KOs associated with degradation of xenobiotics at the protein level. Of the 208 KOs within the database, 4 were identified as part of the creosote feeding functional core. The core KOs unique to creosote feeders coded for the enzymes: enoyl-CoA hydratase (K01692), 4-hydroxy-benzoate decarboxylase (K01612), benzoyl-CoA reductase subunit B (K04113), and 2-pyrone-4, 6-dicarboxylate lactonase (K10221). The three most abundant KOs in creosote feeders were associated with biosynthesis or metabolism of pyrimidines and purines (Fig. 4B). The next most abundant KO coded for 4-carboxymuconol-actone decarboxylase, an enzyme involved in the degradation of benzoate (Fig. 4B). More than half of the KOs (106) were shared across the functional core of creosote and non-creosote feeders with no hierarchical clustering of functional profiles by diet type (Fig. 4A, B; Table S11). Principal component analysis of the KOs at the xenobiotic degradation protein level did not show separation of the functional profiles of woodrat gut microbial communities by diet (Fig. 5A). In addition, there were no xenobiotic degradation KOs identified as significantly enriched in creosote feeders compared to non-creosote feeders (DESeq2).

Co-assembly of the short-read data resulted in 271 total MAGs. These MAGs primarily belonged to the families *Muribaculaceae* (124), *Lachnospiraceae* (48), and *Ruminococcaceae* (15; Tables S12 and S13). Only one MAG belonged to the creosote-feeding core microbiome. This MAG was classified in the family *Treponemataceae* and has an estimated 447 KOs identified, some of which, such as the multi-drug resistance transporters, could enable microbial survival and growth in a high toxin environment (Table S14). No MAGs were significantly enriched in creosote feeders compared to non-creosote feeders (DESeq2). In addition, there was large overlap of the identified functional profiles of MAGs within creosote and non-creosote feeders (Fig. 5B). However, 367 microbial genes were significantly more abundant in creosote feeders (Table S15). When blasted against the UniRef100 database, we found that these differentially abundant genes with an existing KO classification were associated with a wide range of functions with the majority coding for enzymes or cellular transporters, specifically ABC transporters (Table S15). None were identified as being involved in xenobiotic degradation.

Discussion

Diet heavily influences the mammalian gut microbiome [27, 64, 76, 118]. For mammalian herbivores especially, diet should exert strong selection on the gut microbial

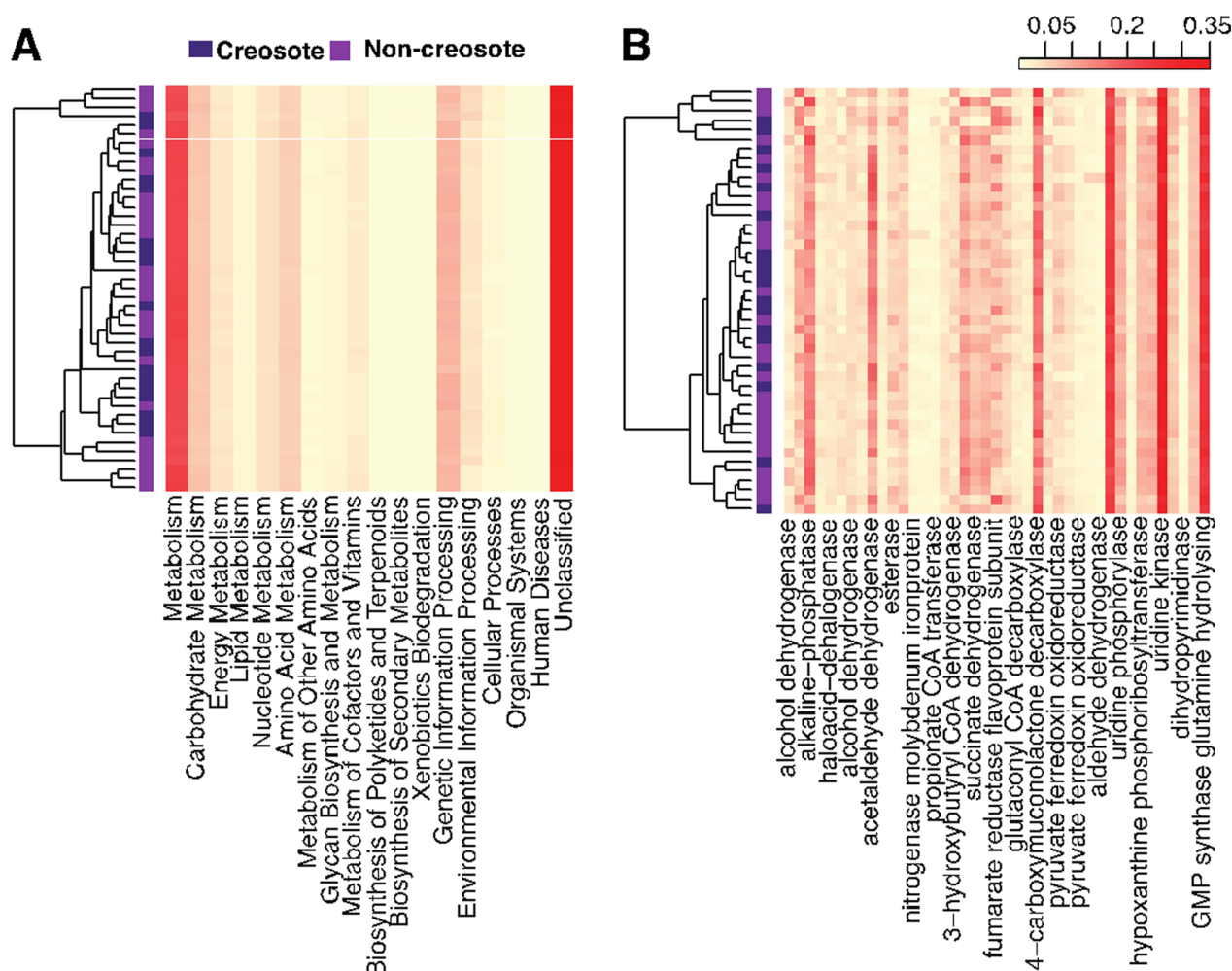Stapleton *et al. Animal Microbiome*   (2024) 6:43

Page 10 of 17

Fig. 4 There was no difference in the functional profiles of gut microbial communities in creosote and non-creosote feeding woodrats using unassembled reads. Heatmaps of relative abundances of the most abundant KEGG pathways (**A**) and the most abundant KEGG-assigned xenobiotic degradation proteins (**B**) in both creosote and non-creosote feeding animals. The dendrogram left of each heatmap represents hierarchical clustering of creosote (dark purple) and non-creosote (light purple) individuals based on Bray–Curtis dissimilarity of KEGG pathway or xenobiotic degradation protein abundance counts. See Tables S11 and S12 for full relative abundance information

community for microbes that provide ecologically relevant functions across hosts, or core microbiota. Little investigation has been done on whether diet exerts this selection for shared microbial taxa and/or functions. Here, we addressed this gap in our knowledge by investigating the taxonomic and functional core microbiome in wild woodrats consuming the same toxic diet. We found core microbes unique to woodrats that consume creosote, and also unique core communities across different gut regions that may aid the host in subsisting on an herbivorous diet. We also identified a functional core unique to creosote feeding woodrats consisting of several KOs that may be involved in the degradation of PSCs.

Based on taxonomy, creosote feeding woodrats harbored distinct gut microbial communities and core

microbes in the foregut, cecum, and hindgut. This is consistent with previous studies that also found that microbial communities differ between the midgut and hindgut in various herbivorous hosts [56, 65, 69, 91]. These gut regions carry out different functions within the host and these physiological differences present unique environments for microbes which likely shape the gut microbial community. The cecum, for instance, is a large fermentation chamber that houses high densities of microbes that ferment dietary fiber [63, 69]. Indeed, we found that the cecum had the highest diversity of microbes and the largest creosote-feeding core microbiome. In addition, most core microbes belonged to the families *Lachnospiraceae* and *Oscillospiraceae*, families known for fiber fermentation in the rumen [2] and previously identified
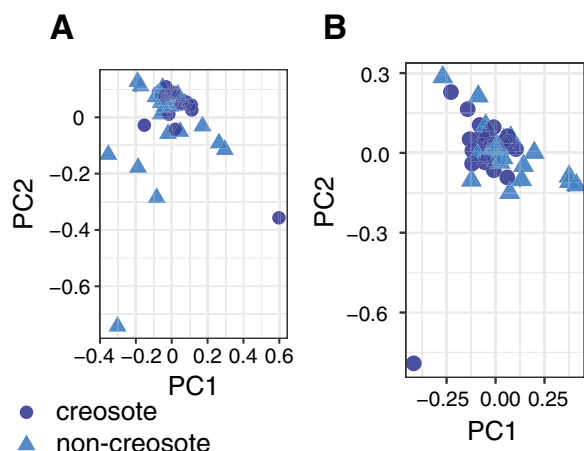
A

B

Fig. 5 There was large overlap of gut microbiome functional profiles between creosote and non-creosote feeders. Principal component analysis of Bray–Curtis distances generated from KO counts at the protein level for **A** unassembled reads and **B** assembled MAGs

as part of the ruminant core microbiome [46]. The large cecum core microbiome of woodrats resembling that of ruminant herbivores may indicate that these taxa are widespread across, and functionally important for, fiber degradation in many mammalian herbivores. Future work could investigate this pattern across even more diverse hosts that consume diets high in fiber, such as reptiles and birds, to determine if the same microbial taxa have been selected for, as previously hypothesized [25].

In contrast to the cecum, the foregut is a small, sacculated region thought to house microbes capable of using PSCs as substrates [67, 85]. The foregut had the largest number of core members belonging to the family *Eggerthellaceae*. Notably, several microbes that belong to *Eggerthellaceae* are capable of degrading PSCs [7, 9, 11, 43, 72]. These microbes may be metabolizing PSCs within the foregut prior to absorption in the small intestine. More core members were identified as *Eggerthellaceae* in the foregut than the cecum; however, this family was ubiquitously identified as part of the core microbiome across gut regions. Other core families in the foregut were either families commonly found in the woodrat gut microbiome (*Lactobacillaceae, Muribaculaceae, Desulfovibrionaceae*) or known fiber degraders (*Lachnospiraceae, Oscillospiraceae*). Retention time of food in the foregut is not long enough for extensive fermentation [69], but, as it precedes the cecum in the gastrointestinal tract, it is possible some fermentation of simple sugars and volatile fatty begins in this region [69].

The foregut microbiome was less diverse than the cecum, with the core microbiome consisting of ~16% as many ASVs as that of the cecum. This lower diversity

may stem from higher concentrations of PSCs in undigested diet, which can reduce microbial growth [26], as well as a low pH (~4.5) which may make the foregut inhospitable to some gut microbiota [61]. Alternatively, the cecum may harbor a large, and taxonomically diverse core microbiome due to the diversity of fibers, waxes, and pectins found in a plant-based diet. Creosote contains 2–3 times more fiber than resin by dry mass, and different plant species produce various kinds of fibers which may require different microbes to break down [84]. The smaller, more taxonomically varied core in the foregut may indicate that the ability to degrade PSCs is not unique to a few microbial taxa, but is possibly a conserved function across microbial lineages.

The hindgut also harbored a smaller size core than the cecum i.e., about 24% that found in the cecum core. Many of these core microbes belong to families also identified as core in the cecum, such as *Muribaculaceae, Oscillospiraceae,* and *Lachnospiraceae*. It is possible that the hindgut core retained fewer members than the cecum because there is little selection from creosote resin once digesta has reached the colon as most of the resin components would be absorbed in the small intestine or fermented by cecal microbes. In addition, we used fecal samples to represent the hindgut and though the feces are often used as a proxy for the hindgut, these microbial communities can be similar or discordant, depending on the host and sampling methods [96, 112, 116]. Some of this variation may stem from the fact that fecal microbial communities may shift after defecation or become contaminated by the environment. In some mammalian species, using feces as a proxy for the hindgut obscured ecological and phylogenetic signals [52]. Though a previous study found that greater than 80% of microbes from woodrat feces collected aseptically were retained in feces collected from a trap [68], even this loss of native microbes may impact the number of shared core microbes found using fecal samples.

Sample size affected the number of core members identified. We collected far more fecal samples than cecum and foregut samples because fecal sample collection is non-invasive. This difference in sample size altered estimates of the core microbiome. When hindgut samples were reduced to the same sample sizes as the other regions, the number of core microbiota increased, though the hindgut microbial community still harbored a smaller core than the cecum. In addition, different families were identified as belonging to the core in the smaller dataset than in the full data set. This has important implications for core microbiome studies as the number of samples used could artificially inflate or shrink the number of microbes identified as core, and even miss taxa of ecological importance. Differential sequencing depth has

Stapleton *et al. Animal Microbiome*   (2024) 6:43

Page 12 of 17

a similar effect where analyses at different depths identify different core microbiota within different taxonomic groups [95]. While sample size can be difficult to keep consistent in studies of the microbial communities of wild organisms, we caution researchers to take this into consideration when designing core microbiome studies or comparing across studies.

Core microbes were more abundant than other ASVs, enriched in creosote feeding woodrats, and occurred more frequently than would be predicted by chance. While the core microbes did not make up a majority of the gut microbiome, they were more abundant than other ASVs and did represent a cumulative relative abundance of > 15% in the foregut and the cecum. This finding is consistent with other studies that classified their core microbiome at the ASV level [1, 104]. In addition, several core microbes were enriched in creosote feeders and identified as overrepresented by neutral models. These overrepresented core members were taxonomically widespread, indicating that these taxa may be beneficial and are potentially selected for within a host [17]. Indeed, several of these enriched or selected for core microbes belonged to families that can provide useful functions to an herbivore such as fiber fermentation (*Ruminococcaceae*) or degradation of PSCs (*Eggerthellaceae*). Taken together, these results suggest that the selected for core members are keystone taxa [6]. Our samples were collected from a large number of geographically distant populations and two different species, factors which significantly influence the community composition of the gut microbial community [114]. Despite samples coming from two species and populations occurring over wide geographic distances (as far as ~ 700 km between creosote feeders), we identified a core microbiome only found in animals consuming creosote, further signifying the possible importance of these cecum and foregut microbes to their host. Experimental manipulation or sequencing of the genome of potential keystone taxa may further elucidate the roles these microbes play in the host gut microbiome.

Some KOs identified as belonging to the functional creosote-feeding core microbiome were related to the metabolism of PSCs. Three of the identified core KOs (4-hydroxybenzoate decarboxylase, benzoyl-CoA reductase subunit B, and 2-pyrone-4, 6-dicarboxylate lactonase) coded for enzymes that play important roles in metabolism of lignans and aromatic rings [12, 14, 48]. Creosote resin is composed of many phenolics, primarily NDGA [101], a lignan with aromatic rings. Therefore, these proteins in the core may play a role in degrading creosote PSCs in the woodrat gut. This finding is consistent with previous work, and warrants further investigation into the particular microbial taxa and pathways that

could metabolize creosote resin. We also identified several KOs coding for ABC transporters that were significantly more abundant in the gut microbial communities of creosote feeders compared to non-creosote feeders. Microorganisms use ABC transporters to efflux toxins, including PSCs, to protect the cell from detrimental effects [35, 119]. Thus, microbes in the creosote-feeding gut microbial community may be using transporters to efflux creosote toxins out of the cell. Indeed, the only core MAG had several KOs related to multi-drug resistance, which are usually transporter genes.

When characterizing the functional profile of the gut microbiome of woodrats, nearly half of the metagenomic data was not assigned to a functional pathway, severely limiting our ability to detect a functional core. It is possible that there exists a larger functional core microbiome in creosote feeding woodrats than we were able to observe due to these pathways being largely uncharacterized. One of the major advantages of metagenomic sequencing is that, in addition to taxonomic information, it provides information on the genomic content of microbes found in a region of interest. Indeed, the main goal of most studies utilizing metagenomic sequencing, including ours, is to characterize the metabolic capacity of microbial communities. Metagenomics is often touted as providing more in-depth and accurate results in comparison to 16S rRNA sequencing and this has been demonstrated in well studied systems, such as the human microbiome where microbial functions have been established [34, 47, 59, 75]. However, poorly studied hosts living in natural environments, much of the genetic information recovered cannot be classified with currently available databases, as was the case in this study and many others [38, 49, 97, 107, 117]. This highlights the need for the sequencing and incorporation of more diverse, wild systems in order to access the wealth of novel, microbial functions that currently remain unknown.

In both unassembled reads and assembled MAGs, we identified a creosote-feeding functional core microbiome, however, a majority of the xenobiotic degradation KOs were shared between creosote and non-creosote feeding woodrats. This result could indicate that enzymes capable of xenobiotic degradation are more pervasive across the woodrat gut microbiota than predicted. These enzymes could be conserved across gut microbiomes as they often perform other essential services such as nutrient synthesis. In addition, while not all woodrats consume creosote bush, all woodrats subsist on natural diets that contain PSCs [67]. Therefore, although they are consuming different plant species, these animals may be exposed to similar suites of PSCs. The woodrats in

Stapleton *et al. Animal Microbiome*   (2024) 6:43

Page 13 of 17

the non-creosote populations feed on plants from the families *Krameriaceae, Fagaceae, Polygonaceae, Ephedraceae,* and *Salicaceae*, all of which can produce phenolics-the predominant class of PSCs in creosote resin [4, 5, 51, 54, 60, 89, 102]. Eating a diet that contains any phenolics may select for similar microbial functions, regardless of phenolic structure or abundance. Further evidence for this notion is that, using our neutral models, we did not see increased selection of the microbiota of creosote feeders compared to that of non-creosote feeders. This result may indicate that all woodrat diets exert selective pressure on gut microbiota for microbes that utilize the particular resources ingested by the host [114]. Also, the functional pathways involved in the degradation of PSCs are not well understood. It is possible that many of the same microbial proteins or protein families are involved in metabolizing diverse arrays of PSCs, making them more universally prevalent in hosts consuming toxic diets [73]. Much work has been done to investigate the effect of diet on the structure, composition, and functional profile of the microbiome in domestic herbivores [23, 24, 41]. Comparatively little work has been done on the interplay of PSCs and gut microbial communities, despite the fact that nearly all plants defend themselves with a wide array of these toxins [29, 115]. Future work could focus on how these toxic compounds affect the microbiome and which microbial enzymes and functional pathways are involved in this process.

In conclusion, this work presents a detailed characterization of the unique taxonomic and functional core microbiome of herbivorous mammals feeding on the same toxic diet. Our work demonstrates that there are core microbes and microbial functions found only in populations of woodrats consuming this toxic diet that are not present in other woodrat populations of the same species that consume different diets. In addition, our work advances our knowledge of which microbes and microbial functions may be involved in degradation of these naturally occurring PSCs present in all herbivore diets. Most of the metagenomic data was not able to be classified, suggesting that much remains unknown about the functional profile of the gut microbiome of wild herbivores and highlights the need for further studies. Investigating functional pathways and microbes capable of breaking down these PSCs may better our understanding of the importance of the gut microbiota to an herbivorous host.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s42523-024-00330-7.

Supplementary Materials

Author contributions
TES conceptualized the project, conducted the fieldwork, conducted the wet bench work, conducted the 16S and trnL sequencing analysis, contributed to the shotgun metagenomic sequencing analysis, wrote the manuscript, and created all figures. LML conducted the shotgun metagenomic sequencing analysis and contributed to the manuscript. HS supplied funding to LML, MDD conceptualized the project, contributed to the writing of the manuscript, and supplied funding to the project.

Availability of data and materials
Raw sequence data associated with this project are accessible through the NCBI Sequence Read Archive under BioProject PRJNA875083. Code for analyses conducted for this study can be found at the following: https://github.com/tess-stapleton/neotoma-creosote-core.

Declarations

Ethics approval and consent to participate
Animal use and procedures were approved by the University of Utah IACUC (16-02011). Animal samples were collected under permits from California (SC-8123), Utah (1COLL5194-1, 2), and Nevada (333663) issued to MDD and California Department of Fish and Wildlife permit SC-2105 issued to Jim Patton.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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Stapleton *et al. Animal Microbiome*   (2024) 6:43

Page 14 of 17

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Stapleton *et al. Animal Microbiome*   (2024) 6:43

Page 15 of 17

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Stapleton *et al. Animal Microbiome* (2024) 6:43

Page 17 of 17

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