Taxonomic differences of gut microbiomes drive cellulolytic enzymatic potential within hind-gut fermenting mammals

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Abstract

Host diet influences the diversity and metabolic activities of the gut microbiome. Previous studies have shown that the gut microbiome provides a wide array of enzymes that enable processing of diverse dietary components. Because the primary diet of the porcupine, Erethizon dorsatum, is lignified plant material, we reasoned that the porcupine microbiome would be replete with enzymes required to degrade lignocellulose. Here, we report on the bacterial composition in the porcupine microbiome using 16S rRNA sequencing and bioinformatics analysis. We extended this analysis to the microbiomes of 20 additional mammals located in Shubenacadie Wildlife Park (Nova Scotia, Canada), enabling the comparison of bacterial diversity amongst three mammalian taxonomic orders (Rodentia, Carnivora, and Artiodactyla). 16S rRNA sequencing was validated using metagenomic shotgun sequencing on selected herbivores (porcupine, beaver) and carnivores (coyote, Arctic wolf). In the microbiome, functionality is more conserved than bacterial composition, thus we mined microbiome data sets to identify conserved microbial functions across species in each order. We measured the relative gene abundances for cellobiose phosphorylase, endoglucanase, and beta-glucosidase to evaluate the cellulose-degrading potential of select mammals. The porcupine and beaver had higher proportions of genes encoding cellulose-degrading enzymes than the Artic wolf and coyote. These findings provide further evidence that gut microbiome diversity and metabolic capacity are influenced by host diet.

Introduction

The microbiome supports animal digestion by detoxifying and breaking down indigestible compounds [1]. There is accumulating evidence that microbial enzymes responsible for these processes are highly conserved across species, and strongly influence overall composition of the microbiome [2]. Intensive study of the human gut microbiome provides a framework for investigating the phylogenetic diversity of wildlife gut microbiomes [2–14]. Similar to what
has previously been observed in human microbiomes [15], the gut microbial diversity of beavers [10], ruminant mammals [14], and giant pandas [16], is strongly influenced by diet. This provides ample rationale for exploring the gut microbiome as a genetic repository of novel enzymes involved in processing diverse dietary components. Animal gut microbiomes provide a potentially rich source of novel microbial genes that can be leveraged for bioengineering applications, including breakdown of complex plant-derived materials [17].

Lignocellulose, a component of the plant cell wall, is an attractive low-cost substrate for biofuel production via microbial fermentation [10]. Before lignocellulose can be fermented, it must be broken down into component sugars by cellulases, hemicellulases, and various debranching enzymes [18]. The porcupine, *Erethizon dorsatum*, is an herbivore that feeds on lignified plants, coniferous (preferred) and deciduous cambium (inner bark), and flowers [19]. As a hindgut fermenter, the porcupine has an enlarged cecum where microbial digestion is largely confined [20]. Because the primary diet of porcupines is lignified plant material, we hypothesized that the porcupine microbiome would be replete with enzymes required to degrade lignocellulose.

The primary objective of this study, completed by undergraduate students of the Dalhousie iGEM team, was to characterize the gut microbiome of the porcupine along with 20 other species (within the Rodentia, Carnivora, and Artiodactyla orders) from the Shubenacadie Wildlife Park in Nova Scotia, Canada. All species were profiled using 16S rRNA gene sequencing. Mammals in the same order had more similar microbiome profiles compared to those with differing taxonomic classification. Metagenomic shotgun sequencing was conducted on select representative herbivores (porcupine, *Castor canadensis* (beaver)) and carnivores (*Canis latrans* (coyote), *Canis lupus arctos* (Arctic wolf)). In these four selected mammals, dramatic bacterial taxonomic differences were detected using both 16S and metagenomic sequencing methods. Importantly, the genes encoding cellulolytic enzymes were significantly enriched in porcupine and beaver microbiomes. Taken together, these findings confirm that the microbiomes of hind-gut fermenters are a rich source of cellulose-degrading enzymes.

### Methods

#### Data availability

Quality controlled and processed metagenomic and 16S rDNA sequence data are available through the Sequence Read Archive (NCBI) at accession number SRP115632 and SRP115643 respectively.

#### Animal containment and diet

All 21 animals were sampled from Shubenacadie Wildlife Park. Animals lived in outdoor pens modeled after their natural habitats. For instance, roaming animals, such as deer and elk, were held in large pastures with unlimited access to grass. Animals were co-housed regardless of whether they were born in captivity or rescued from the wild. All animals received diets that met their species-specific requirements (Table 1).

#### DNA extraction and sequencing

Fecal samples from 21 different mammals at Shubenacadie Wildlife Park were collected by park staff and stored at -80°C [21]. DNA was extracted using a Mobio PowerFecal® extraction kit (QIAGEN, Germany) according to the manufacturer’s protocol. DNA purity was assessed using a Nanodrop spectrophotometer (NanoDrop Technologies Inc., USA). DNA samples were submitted for 16S rRNA gene sequencing at the Integrated Microbiome Resource (IMR).
at Dalhousie University. 16S rRNA sequencing primers (Table 2), provided by the IMR, were modified from the primers used by Comeau et al. to improve compatibility with the Illumina platform [22]. Variable regions V6-V8 of the bacterial 16S rRNA gene were amplified from all purified DNA samples and sequenced on an Illumina MiSeq using paired-end 300 bp

**Table 1. Diets of the 21 mammals sampled from Shubenacadie Wildlife Park.** Presented is the animal common name, the daily diet, and the speciality food stuffs required to meet dietary requirements.

| Animal      | Daily Diet                                                                 | Speciality Foods (Not provided daily)                                                                 |
|-------------|----------------------------------------------------------------------------|-------------------------------------------------------------------------------------------------------|
| Arctic fox  | 100 g Pacifica dry dog food, 100 g wild prairie dog food,                  | 250 g whole rabbit, hard-boiled egg, 25 g berries and fruit, 200 g ground chicken, 200 g lean horse  |
| Arctic wolf | 1.8 kg canine diet, 2 cups of large breed dog food                        | 2.2 kg ground chicken, hard-boiled egg, 2 kg whole rabbit, and 1.25 kg of beef knuckles              |
| Beaver      | 200 g alfalfa, 250 g rabbit chow, 35 g chopped carrots, 35 g chopped apple,| unlimited branches (alder, maple, birch, and poplar)                                                |
| Black bear  | 2 kg canine plus large breed adult, hard-boiled egg, 75 g berries and fruit, 250 g chopped yam, 35 g chopped broccoli | 2 kg canine diet, fish oil, 2 kg whole herring, 75 g of berries and fruit                           |
| Elk         | Browser pellets, Purina deer pellets, Timothy hay, salt block             | pasture grazing                                                                                     |
| Fisher      | 300 g small carnivore diet, fish oil                                      | 300 g ground chicken, 25 g berries or fruit, 300 g lean horse meat, 300 g whole rabbit             |
| Mink        | 100 g otter mixture                                                       | 80 g whole turkey, 20 g berries or fruit                                                            |
| Moose       | Mazuri moose maintenance-free choice @ 100%, 1 kg cut-up browse (Birch, Willow, Alder, Maple, Aspen and Balsam Fir) leaves and small branches—1/4” or less in diameter, 20 g equine plus supplement, salt block | pasture grazing                                                                                     |
| Pine martin | 150 g small carnivore diet                                                | 150 g ground chicken, 25 g berries or fruit, 150 g turkey                                          |
| Porcupine   | 250 g rabbit chow, 100 g alfalfa, 15 g of each; chopped carrot, chopped apple, potato, fruit and berries, salt block | free access to softwood trees, antler, free access to sod                                           |
| Raccoon     | 150 g Pacifica dry food, 150 g prairie dry dog food, 40 g berries and fruit, 40 g chopped apple | hard-boiled egg, 250 g small carnivore diet                                                          |
| Red deer    | Free choice; Browser pellets, deer pellets, Timothy hay, salt block       | free range                                                                                           |
| Striped skunk | 35 g dry dog food, 35 g prairie dog food, 20 g apple, 20 g fruit or berries | hard-boiled egg                                                                                     |
| Swift fox   | 75 g dry dog food, 75 g prairie dog food, 25 g berries or fruit          | 150 g whole rabbit, 150 g small carnivore diet, hard-boiled egg, 150 g ground chicken, 150 g lean horse |
| Coyote      | 800 g canine diet, 1 cup large breed dry dog food, fish oil               | 900 g chicken necks, hard-boiled egg, 1 kg whole rabbit, 750 g beef knuckles, vitamin supplement    |

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Table 2. List of oligonucleotides used for 16S rDNA amplification.

| Name of Primer | Sequence | Description                      |
|----------------|----------|----------------------------------|
| Forward (V6)   | ACGGGHNNACCTTACC | Forward Primer for 16S rDNA gene |
| Reverse (V8)   | ACGGGCRGTGWGTRCAA | Reverse Primer for 16S rDNA gene |

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sequencing [23]. For metagenomic shotgun sequencing, extracted DNA from coyote, porcupine, Arctic wolf and beaver samples was PCR-amplified using primers from Nextera following the Nextera XT (Illumina) protocol. Then, sequencing libraries were prepared from purified PCR products (1 ng) using the Nextera XT Library Preparation Kit (Illumina). Libraries were cleaned-up and normalized using the Just-a-Plate 96 PCR Purification and Normalization Kit (Charm Biotech). Complete libraries were then pooled and sequenced in a portion of a 300+300 bp Pair-End MiSeq run (Illumina 600-cycle v3 kit).

### 16S sequencing data analysis

Analysis of 16S rRNA sequence data was performed as previously described using Microbiome Helper [23]. Samples from each of the 21 mammals were sequenced only once, with exceptions in the beaver, porcupine, Arctic wolf and coyote which were sequenced in biological triplicate. Raw data in FASTQ format from Illumina sequencing was analyzed for quality via FASTQC. Paired-end reads were stitched together using PEAR [24]. Stitched reads were filtered for quality (q-score >30 over at >90% of the read) and to ensure forward and reverse primers matched using FASTX-toolkit and BBMAP. FASTQ files were converted to FASTA format and all sequences containing a variable nucleotide ‘N’ were removed. Chimeric reads were removed using VSEARCH [25]. Taxonomic assignments were made by matching sequences into Operational Taxonomic Units (OTUs) with 97% sequence identity and comparing to the Green-Genes 16S rRNA database using QIIME [26–27]. Samples were normalized for differences in sequencing depth using DeSeq2’s negative binomial distribution [28] and principle coordinate analysis was performed using UniFrac beta-diversity through QIIME [29].

KEGG Orthologs (KO) [30–32] were inferred using PICRUSt to estimate functional-gene profiles from the triplicate porcupine, beaver, coyote, and Arctic wolf triplicate 16S sequencing data [33]. Differences in KO relative abundances across species were analyzed using STAMP, and subjected to an ANOVA analysis with Benjamini-Hochberg FDR correction to generate the predicted mean proportion of sequences and the box-plots [34].

### Metagenomic sequencing data analysis

Raw sequences were inspected for overall quality using FASTQC and then stitched using the program PEAR [24], followed by screening for potential contaminant sequences from human and spiked-in PhiX using BowTie2 [35]. We opted to only use stitched reads to avoid artificial annotation due to short read fragments, and to ensure higher quality annotation. After removal of contaminant data, the software program Trimmomatic was used with default quality cut off based on FASTQC inspection [36]. MetaPhlAn2 was used to screen the data for taxonomy, and assign reads to bacterial species [37]. Following taxonomic assignment, DIAMOND and HUMAnN1 were used to assign functionality and metabolic profiles to the sequenced metagenomes. DIAMOND was used to perform the initial Pre-HUMAnN1 search against the KEGG database with a default e-value cutoff of 0.001 (stringent selection for protein matches) [38]. The HUMAnN1 software tool was used to get a functional profile of the metagenomic sequences which was then converted to STAMP format [39]. STAMP was used to perform an ANOVA analysis with Benjamini-Hochberg FDR correction and to generate box plots for comparison to the PICRUSt analysis from the 16S rRNA sequencing above. The data output from MetaPhlAn2 was visualized using GraPhlAn, after conversion of the MetaPhlAn2 output data with the Export2GraPhlAn scripts. Summary heat maps of the HUMAnN1 output were generated using Morpheus hierarchal clustering with a one minus Pearson correlate matrix and an average linkage method [40]. All four animals were sequenced in triplicate.
HUMAnN2 was run on the metagenomic dataset to confirm the HUMAnN1 results and to identify taxa likely contributing to the gene families of interest in each sample [38]. HUMAnN2 was run in uniref-50 mode using the same KEGG database as used for HUMAnN1 analysis. HUMAnN2 gene family abundance data was analyzed using a two-way ANOVA with the Holm-Sidak multiple test correction.

Results

Captive mammals display overlapping microbiome composition

Illumina sequencing of the 16S rRNA gene (V6-8 region) for the 21 Shubenacadie Wildlife Park mammals produced a total of 1,002,901 paired-end overlapping reads (Table 3). After merging paired-end reads and filtering for sequences longer than 400 base pairs (90% read-quality of >30), samples were left with at least 84% of their initial number of sequences. The number of OTUs identified per sample at 97% sequence identity when clustering ranged from 786 (Skunk) to 5,310 (Red Deer). Alpha rarefaction curves confirmed OTU saturation for most of the sequences, although the porcupine, moose, and red deer samples appeared to have less saturated sequencing than the others (S1 Fig).

The 21 mammalian microbiomes were then compared using weighted unifrac beta-diversity analysis of the 16S rRNA sequencing data (Fig 1). The principal component analysis showed that mammals in the same order (Carnivora, Rodentia, Artiodactyla, etc.) had more similar microbiomes compared to those from different orders. The order Carnivora was observed twice in the beta-diversity plot representing two different clusters of Carnivora: the

Table 3. Summary of profiled species and 16S rRNA sequencing details. Presented is the common animal name, the scientific animal name, the total number of sequences, the number of OTUs per sample, number of unique OTU’s per sample, and the number of remaining sequences after stitching and quality filtering.

| Host Animal   | Scientific Name               | OTU’s identified per Sample | Unique OTU’s | Total Sequences | Sequences after Pairing/Quality Filtering |
|---------------|--------------------------------|------------------------------|--------------|----------------|-------------------------------------------|
| Red Fox       | Vulpes vulpes                 | 970                          | 283          | 50,870         | 43,120                                   |
| Domestic Rabbit| Oryctolagus cuniculus         | 1,353                        | 995          | 39,559         | 33,598                                   |
| Otter         | Lontra canadensis             | 881                          | 425          | 61,242         | 52,578                                   |
| Coyote        | Canis latrans                 | 1,275                        | 625          | 50,496         | 42,765                                   |
| Mink          | Neovision vision              | 1,082                        | 660          | 60,591         | 52,126                                   |
| Skunk         | Mephitis mephitis             | 786                          | 328          | 55,787         | 47,833                                   |
| Raccoon       | Procyon lotor                 | 850                          | 400          | 44,213         | 37,857                                   |
| Black Bear    | Ursus americanus              | 1,152                        | 622          | 49,409         | 42,604                                   |
| Porcupine     | Erethizon dorsatum            | 3,350                        | 2,894        | 20,933         | 17,599                                   |
| Elk           | Cervus canadensis             | 2,144                        | 1,104        | 19,599         | 16,546                                   |
| Fisher        | Pekania pennanti              | 793                          | 308          | 64,867         | 56,003                                   |
| Arctic Fox    | Vulpes lagopus                | 2,296                        | 1,178        | 55,170         | 46,954                                   |
| Snowshoe Hare | Lepus americanus              | 1,560                        | 1,157        | 64,785         | 54,949                                   |
| Marten        | Martes americana              | 814                          | 316          | 82,374         | 70,689                                   |
| Beaver        | Castor canadensis             | 2,155                        | 1,920        | 54,564         | 46,209                                   |
| Swift Fox     | Vulpes velox                  | 923                          | 172          | 47,605         | 40,081                                   |
| Arctic Wolf   | Canis lupus subs. arctos      | 1,354                        | 657          | 34,253         | 28,954                                   |
| Red Deer      | Cervus elaphus                | 5,310                        | 3,553        | 24,662         | 21,805                                   |
| Moose         | Alces alces                   | 3,997                        | 2,771        | 27,640         | 23,336                                   |
| Timber Wolf   | Canis lupus                   | 917                          | 314          | 45,391         | 38,354                                   |
| Ground Hog    | Marmota monax                 | 2,301                        | 1,640        | 47,891         | 41,039                                   |

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Canidae family (including foxes, wolves, and coyotes), and the Musteloidae superfamily (including the fisher, skunk, otter, mink, marten, and bear) [41–42]. The two distinct clusters indicated that the microbiomes of the Carnivora (super)families were divergent, and warranted separate locations on the beta-diversity plot. Next, the specific composition of the microbiome was analyzed through the 16S data of the sampled animals.

Bacteroidales was the most prominent order isolated from both the porcupine and the beaver were (66.0%, 59.6% respectively; Fig 2). The next most abundant order in the porcupine was Clostridiales (27.7%), whereas the next most abundant order in the beaver was Verrucomicrobials (3.1%) (Fig 2). A significant number of bacteria were unassigned in both the porcupine and beaver datasets (2.8%, 26.8%), suggesting either the presence of novel species within these hosts and/or poor quality of reads (Fig 2). Like the porcupine and beaver, the microbiomes from the Arctic wolf, swift fox, Arctic fox, coyote, timber wolf, and red fox contained a large percentage of Bacteroidales (26.0%, 56.3%, 35.6%, 18.5%, 53.7% and 46.4%) and Clostridiales (52.2%, 11.5%, 29.1%, 37.5%, 26.3% and 37.0%), while containing two other abundant orders: Fusobacteriales (19.0%, 27.6%, 29.3%, 37.5%, 26.3% and 36.9%) and Burkholderiales (0.4%, 1.2%, 0.7%, 0.2%, 3.7% and 2.0%; Fig 2). The most abundant orders found in the Artiodactyla order (Elk, Moose, and Red Deer) were: Bacteroidales (58.1%, 36.5% and 26.3% respectively), Bacillales (3.6%, 15.6%, and 0.5%) and Spirochaetales (2.0%, 0.1%, and 15.1%; Fig 2). In
the Artiodactyla order (Elk, Moose, and Red Deer), Clostridiales was less abundant (0.5%, 2.9% and 5.1%; Fig 2). For the mammals in the Mustelidae order (Marten, Skunk, Mink, Fisher, and Otter), the most abundant bacterial order was Clostridiales (5.1%, 32.1%, 14.2%, 92.8%, and 5.0%) followed by Pseudomonadales (28.0%, 11.1%, 37.8%, 0.5%, and 42.8%) and Enterobacteriales (62.3%, 38.1%, 0.2%, 2.6%, and 14.2%; Fig 2). Finally, there were five mammals—the raccoon, domestic rabbit, snowshoe hare, bear, and groundhog—that did not group into the Carnivora, Rodentia, or Artiodactyla orders. Bacteroidales was the most abundant bacterial order in the raccoon, domestic rabbit, and snowshoe hare (40.1%, 38.1%, and 59.8% respectively), whereas Clostridiales was most abundant in the bear (36.8%), and Flavobacteriales was most abundant in the groundhog (36.6%; Fig 2). Clostridiales was also abundant in the raccoon and snowshoe hare microbiomes (23.0%, and 33.1%, respectively), but scarce in the domestic rabbit and groundhog (1.2%, and 1.3%, respectively; Fig 2). The complete taxonomic assignments for 16S rRNA sequencing datasets can be found in S1 Table.

Further taxonomic characterization was conducted by 16S sequencing, in biological triplicate, on fecal samples from a subset of four mammals: the beaver and the porcupine (cellulose-
consuming mammals), and the coyote and the Arctic wolf (carnivorous mammals). Alpha rarefaction curves confirmed OTU saturation for most of the sequences (S2 Fig). In all four mammals, the most abundant bacterial order was Bacteroidales (52.07\% +/- 3.02\% (porcupine), 58.20\% +/- 5.61\% (beaver), 34.64\% +/- 13.68\% (coyote), and 37.96\% +/- 12.89\% (Arctic wolf). The second most abundant order in the Arctic wolf and porcupine was Clostridiales (30.38\% +/- 16.78\%, and 30.85\% +/- 4.19\%, respectively), whereas in the coyote it was Fusobacteriales (24.33\% +/- 5.41\%), and in the beaver it was unclassified (18.19\% +/- 1.31\%). A summary of the 16 highest orders in each mammalian sample can be found in Table 4, while the complete taxonomic assignment can be found in S2 Table.

Next the genetic repertoire of the microbiome was analyzed using metagenomic sequencing. The porcupine, beaver, coyote, and Arctic wolf samples were sequenced in triplicate to allow statistical analysis. Following sequencing, the mean number of reads were 4,378,272 for the porcupine; 4,599,146 for the beaver; 4,208,902 for the coyote; and 4,137,256 for the Arctic wolf. After stitching, more than 50\% of sequences remained in each sample, and after screening, greater than 43.5\% of the original sequences remained (Table 5).

Further taxonomic characterization of the porcupine, beaver, coyote, and Arctic wolf microbiomes was conducted by analyzing the metagenomic shotgun sequencing data. The microbial taxonomy of the beaver and porcupine microbiomes were similar to each other but diverged from microbiome taxonomies of the Arctic wolf and coyote (Fig 3). Perhaps not surprisingly, the most prevalent bacteria in both groups were Clostridiales (49.46\% and 38.78\% in the cellulose-consuming and carnivorous groups, respectively) and Bacteroidales (22.77\% and 21.08\% respectively). In the cellulose-consumer group, the next most prevalent bacterial orders were: Pseudomonales (13.22\%), Burkholderiales (3.99\%), Methanobacteriales (1.49\%), Selenomonadales (1.42\%), and Sphingobacteriales (1.26\%). In the carnivorous group, the next most prevalent bacterial orders were: Coriobacteriales (14.49\%), Erysipelotrichales (7.82\%), Burkholderiales (4.94\%), Fusobacteriales (4.99\%), and Flavobacteriales (3.72\%). Apart from the most prominent bacteria (Clostridiales and Bacteroidales) and the least prominent (Burkholderiales)

| Bacterial Order       | Arctic Wolf Average (StDev) | Coyote Average (StDev) | Porcupine Average (StDev) | Beaver Average (StDev) |
|-----------------------|----------------------------|------------------------|---------------------------|------------------------|
| Bacteroidales         | 38.0 (+/-12.9)             | 34.6 (+/-13.7)          | 52.1 (+/-3.0)             | 58.2 (+/-5.6)          |
| Clostridales          | 30.4 (+/-16.8)             | 19.6 (+/-16.1)          | 30.9 (+/-4.2)             | 12.8 (+/-5.3)          |
| Fusobacteriales       | 20.9 (+/-8.3)              | 24.3 (+/-5.4)           | 0                         | 2.6 (+/-1.9)           |
| Unassigned            | 7.34 (+/-1.8)              | 18.6 (+/-2.8)           | 10.4 (+/-0.9)             | 18.2 (+/-1.3)          |
| Burkholderiales       | 0.97 (+/-0.5)              | 0.2 (+/-0.1)            | 0.1 (+/-0.1)              | 1.9 (+/-1)             |
| Coriobacteriales      | 0.77 (+/-0.5)              | 1.3 (+/-1.5)            | 0.1                       | 0.3 (+/-0.2)           |
| Pseudomonadales       | 0                         | 0                      | 1.5 (+/-0.9)              | 0.2 (+/-0.2)           |
| Erysipelotrichales    | 0.97 (+/-0.5)              | 0.9 (+/-0.6)            | 0.8 (+/-0.1)              | 1.5 (+/-1)             |
| Aeromonadales         | 0.26 (+/-0.1)              | 0.3 (+/-0.2)            | 0                         | 0.1 (+/-0.1)           |
| RF39                  | 0                         | 0                      | 0.9 (+/-0.5)              | 0.2 (+/-0.1)           |
| Flavobacteriales      | 0                         | 0                      | 0.9 (+/-0.9)              | 0                      |
| YS2                   | 0                         | 0                      | 0.4 (+/-0.1)              | 0                      |
| Enterobacteriales     | 0.1 (+/-0.1)               | 0                      | 0.3 (+/-0.2)              | 0                      |
| Lactobacillales       | 0.2 (+/-0.1)               | 0                      | 0.2 (+/-0.2)              | 0                      |
| Verrucomicrobiales    | 0                         | 0                      | 0.9 (+/-0.9)              | 3.5 (+/-0.7)           |
| RF32                  | 0                         | 0                      | 0.2 (+/-0.1)              | 0                      |

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that appear in both the carnivore and herbivore groups, bacterial composition is distinct. The complete taxonomic assignment for the metagenomic shotgun sequencing can be found in S3 Table.

**Cellulose metabolism genes are more abundant in hind-gut fermenters than carnivores**

Next, the gene family profile was annotated using HUMAnN1 to determine the abundances of KEGG pathways in the four select mammalian samples. The highest mean pathway abundances in the Arctic wolf, coyote, beaver and porcupine samples were: D-glutamine and D-glutamate metabolism (KO00471; 3.27%, 3.16%, 2.90%, 2.73%), ribosome (KO03010; 2.89%, 2.87%, 2.81%, 3.09%), aminoacyl-tRNA biosynthesis (KO00970; 2.50%, 2.39%, 2.45%, 2.71%), valine, leucine and isoleucine biosynthesis (KO00290; 2.43%, 2.32%, 2.81%, 3.03%) and one-carbon pool by folate (KO00670; 2.36%, 2.18%, 2.32%, 2.24%) (Table 6). These pathways were expected to be among the highest as they are core metabolic pathways [43]. Complete functional assignments for metagenomic shotgun sequencing can be found in S4 Table.

A heat-map was then generated to visualize the starch and sucrose pathway families identified from the KEGG database. Here cellulolytic and sugar transport genes were displayed from the metagenomic data from the four animals (Fig 4). The map demonstrated that the porcupine and the beaver had high abundances of cellulose-metabolizing genes and low abundances of transport genes. The opposite result was true for the coyote and Arctic wolf.

Three cellulose-metabolizing genes were identified from the heat map and selected to compare relative gene abundances in the porcupine, beaver, coyote, and Arctic Wolf. The three representative enzymes were: endoglucanase (K01179), cellobiose phosphorylase (K00702) and beta-glucosidase (K05349). These enzymes were used based on their critical role in cellulose metabolism. PICRUSt analysis (using 16S sequencing) predicted a significant enrichment of the mean relative frequencies of endoglucanase and cellobiose phosphorylase in the beaver (0.099%, and 0.015%) and porcupine (0.070%, and 0.011%), in comparison to the Arctic wolf (0.044%, and 0.003%) and coyote (0.035%, and 0.002%) (Fig 5A, p = 5.10e-5, and p = 3.97e-5, respectively). The predicted mean relative frequency of beta-glucosidase was not statistically significant (Arctic wolf (0.21%), coyote (0.23%), beaver (0.14%) and porcupine (0.27%, Fig 5A). Metagenomic analysis was concordant with most of the PICRUSt predictions. For

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**Table 5. Summary of profiled species and sequences from shotgun metagenomic sequencing.** Presented is the number of reads in total, after stitching with PEAR, and after screening to remove human and PhiX reads.

| Host Animal (Scientific Name) | Replicate | Number of Reads | Reads After Stitching | Reads After Screening/Trimming |
|--------------------------------|-----------|-----------------|----------------------|--------------------------------|
| Arctic Wolf (Canis lupus subs. arctos) | 1 | 4,588,281 | 3,436,267 | 3,134,724 |
| | 2 | 2,760,442 | 1,721,568 | 1,655,754 |
| | 3 | 5,063,044 | 4,018,295 | 3,247,880 |
| Coyote (Canis latrans) | 1 | 4,724,246 | 3,312,867 | 3,177,125 |
| | 2 | 3,152,637 | 1,617,376 | 1,563,285 |
| | 3 | 4,749,822 | 3,553,397 | 3,245,236 |
| Beaver (Procyon lotor) | 1 | 4,597,577 | 3,233,205 | 3,053,519 |
| | 2 | 4,491,180 | 3,018,441 | 2,880,856 |
| | 3 | 4,706,681 | 3,641,930 | 3,266,187 |
| Porcupine (Erethizon dorsatum) | 1 | 3,534,138 | 2,243,918 | 2,170,701 |
| | 2 | 4,796,618 | 3,489,595 | 3,311,107 |
| | 3 | 4,805,060 | 3,711,043 | 3,256,940 |

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endoglucanase and cellobiose phosphorylase, there was a significant enrichment of mean relative frequencies in the beaver (0.0781%, and 0.0406%), and porcupine (0.0388%, and 0.0915%), in comparison to the Arctic wolf (0.0212%, and 0.0046%) and coyote (0.0039%, and 0.0259%) (Fig 5B, p = 1.46e-5, and p = 2.72e-7, respectively). The predicted mean relative frequency of beta-glucosidase was not statistically significant (Arctic wolf (0.00093%), beaver (0.0017%), coyote (0.0011%), and porcupine (0.0019%) (Fig 5B).

HUMAnN2 analysis was subsequently used to identify the relative abundance of endoglucanase and beta-glucosidase in the four select mammals (cellobiose phosphorylase was not annotated when using HUMAnN2). The average relative abundance (per kb) of endoglucanase (K01179) was 56.23, 63.83, 216.97, and 303.6 for the Arctic wolf, coyote, beaver, and porcupine, respectively (Fig 6, \( p = 0.0137 \)). The average relative abundance (per kb) of beta-glucosidase (K05349) was 593.16, 861.17, 1116.26 and 1098.92 for the Arctic wolf, coyote, beaver, and porcupine, respectively (Fig 6, \( p = \text{ns} \)). HUMAnN2 enables quantification of taxonomic orders that contribute to enzyme abundance. Here, HUMAnN2 analysis was used to provide a taxonomic breakdown of organisms likely contributing to beta-glucosidase and endoglucanase abundance in the porcupine, beaver, coyote, and Arctic wolf microbiomes (S5 Table). The most abundant organisms contributing the beta-glucosidase gene family to the Arctic wolf, coyote, beaver, and porcupine microbiomes were *Bacteroides* (42.66%, 47.89%, 41.48%, 30.55%), *Bifidobacterium* (8.05%, 5.61%, 8.72%, 11.54%), *Clostridium* (5.84%, 4.85%, 11.96%, 8.55%), *Parabacteroides* (6.86%, 5.08%, 5.80%, 6.29%), and *Butyrivibrio* (5.72%, 3.27%, 5.74%, 7.35%) (Table 7). The most abundant organisms contributing the endoglucanase gene family to the Arctic wolf, coyote, beaver, and porcupine microbiomes were *Clostridium* (22.95%, 25.83%, 48.66%, 42.82%), *Eubacterium* (11.51%, 10.05%, 23.52%, 24.93%), *Methanobrevibacter* (17.49%, 19.13%, 1.23%, 2.18%), *Lactococcus* (4.96%, 8.19%, 3.25%, 13.73%) and *Deinococcus* (9.00%, 10.77%, 4.55% and 3.28%) (Table 8). The entirety of the HUMAnN2 data-set can be found in S5 Table.

Table 6. The 13 most abundant gene pathways identified using HUMAnN1 (metagenomic sequencing) in the Arctic wolf, coyote, porcupine, and beaver. Units are relative abundance of gene pathway sequences expressed as a percentage average. Standard deviation (StDev) is shown in brackets.

| Gene Pathway (KEGG)                  | Arctic Wolf Mean(StDev) | Coyote Mean(StDev) | Porcupine Mean(StDev) | Beaver Mean(StDev) |
|--------------------------------------|-------------------------|--------------------|-----------------------|-------------------|
| D-Glutamine and D-glutamate metabolism | 3.27(+/-0.16)           | 3.16 (+/-0.12)     | 2.73 (+/-0.14)        | 2.9 (+/-0.05)     |
| Ribosome                             | 2.89(+/-0.21)           | 2.87 (+/-0.26)     | 3.09 (+/-0.05)        | 2.81 (+/-0.09)    |
| Valine, leucine and isoleucine biosynthesis | 2.43(+/-0.16)           | 2.32 (+/-0.13)     | 3.03 (+/-0.05)        | 2.81 (+/-0.13)    |
| Aminoacyl-tRNA biosynthesis          | 2.5(+/-0.22)            | 2.39 (+/-0.2)      | 2.71 (+/-0.06)        | 2.45 (+/-0.13)    |
| One carbon pool by folate            | 2.36(+/-0.08)           | 2.18 (+/-0.1)      | 2.24 (+/-0.03)        | 2.32 (+/-0.09)    |
| Thiamine metabolism                  | 2.23(+/-0.05)           | 2.11 (+/-0.02)     | 1.74 (+/-0.03)        | 2.05 (+/-0.04)    |
| Peptidoglycan biosynthesis           | 2.18(+/-0.07)           | 2.16 (+/-0.09)     | 2.03 (+/-0.04)        | 1.98 (+/-0.03)    |
| Alanine, aspartate and glutamate metabolism | 2.13(+/-0.06)           | 2.06 (+/-0.1)      | 2.19 (+/-0.06)        | 2.22 (+/-0.13)    |
| Lysine biosynthesis                  | 1.87(+/-0.09)           | 1.78 (+/-0.03)     | 1.93 (+/-0.02)        | 2.01 (+/-0.01)    |
| Streptomycin biosynthesis            | 1.67(+/-0.1)            | 1.77 (+/-0.03)     | 2.1 (+/-0.23)         | 2 (+/-0.9)        |
| Biotin metabolism                    | 1.89(+/-0.61)           | 2.09 (+/-0.5)      | 1.33 (+/-0.11)        | 1.67 (+/-0.15)    |
| D-Alanine metabolism                 | 1.82(+/-0.2)            | 1.87 (+/-0.07)     | 2.73 (+/-0.06)        | 1.83 (+/-0.08)    |
| Pantothenate and CoA biosynthesis    | 1.75(+/-0.02)           | 1.77 (+/-0.1)      | 1.97 (+/-0.07)        | 2.01 (+/-0.03)    |
Discussion

Here we report high abundance of cellulolytic enzymes in the porcupine gut microbiome. We initially surveyed 21 mammalian microbiomes using 16S rRNA sequencing, and clustered them into taxonomic groups (Fig 1). From this, we identified differences in bacterial composition amongst mammalian orders. We focused our attention on four mammals for in-depth 16S analysis in triplicate: the porcupine and beaver (Rodentia), and the coyote and Arctic wolf (Canidae). Clostridiales and Bacteroidales were abundant in both the Rodentia and Canidae groups. In addition, the Arctic wolf and coyote samples contained Fusobacterium and Burkholderiales, and the beaver and porcupine contained RF39 and Verrucomicrobiales. We observed that not all species grouped within their particular order (ex. the groundhog was...
Fig 5. The comparison between the predicted (PICRUSt) and actual (HUMANN1) mean sequence proportion of genes encoding particular enzymes in selected mammalian microbiomes. Box plots of predicted (A; PICRUSt) or identified (B; HUMANN1) mean
sequence proportion of genes encoding the indicated enzymes are shown for feces of four indicated mammals. Predicted data were obtained via PICRUSt analysis from 16S rRNA sequencing, in triplicate. Mean sequence proportions were obtained via HUMAnN1 analysis from shotgun metagenomic sequencing, in triplicate. Statistical significance was determined using ANOVA with Benjamini-Hochberg FDR correction for multiple tests.

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separate from the rest of the Rodentia order), which may be in part due to the constraints of 16S rRNA sequencing. If we had selected a different 16S rRNA region, or had a larger database of known microorganisms, we may have seen an increased congruence of microbiomes amongst these animals [44]. However, without further sequencing of these microbiomes, we are unable to make any formal conclusions.

We re-sequenced the Arctic wolf, coyote, porcupine, and beaver in biological triplicate via metagenomic shotgun sequencing. Within the Rodentia mammals, Clostridiales and Bacteroidales still had the highest abundance, but Burkholderiales and Pseudomonadales were also prevalent. The Pseudomonadales are known to denitrify organic matter, and are part of the carbohydrate-metabolizing Gammaproteobacteria phylum [45]. A few examples of cellulose-metabolizing Gammaproteobacteria are Acinetobacter, Pseudomonas, and Staphylococcus, which have been isolated from termite gut microbiomes and have been shown to grow when cellulose is the sole carbon source [46]. Our metagenomic sequencing data did not precisely match our 16S rRNA sequencing data; we reasoned that our metagenomic sequencing data was likely more accurate due to previously reported biases in 16S primer sets [47]. In the Canidae mammals, the most abundant bacterial orders were Clostridiales and Bacteroidales, followed by Coriobacteriales, Erysipelotrichales and Burkholderiales. The roles of specific bacterial families in the Canidae gut microbiome may be inferred from human microbiome studies. For example, the Coriobacteraceae family (of the Coriobacteriales order) consists of bacteria that degrade bile salts and steroids [48] and the Erysipelotrichaceae family (of the Erysipelotrichales order) have been linked to lipidemic profiles of human hosts [49]. Thus, the high prevalence of Erysipelotrichaceae in Canidae may be attributed to high fat diets.

![Diagram A](https://doi.org/10.1371/journal.pone.0189404.g005)

**Fig 6.** HUMAnN2 analysis gene family abundance and these gene families contributing taxa. Whisker plots for the relative abundance (measured in reads mapped per kilobase) were generated from HUMAnN2 functional analysis for endoglucanase (K01179) and beta-glucosidase (K05349). A two-way ANOVA using Holm-Sidak multiple test correction we used to test significance (A, \( p = 0.0137 \); B, not significant). The taxonomic breakdown for each gene family was also generated using HUMAnN2 output and presented at the genus level (C).

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Beavers and porcupines are monogastric hindgut fermenters that rely on symbiotic gut bacteria to break down plant material. Fermentation is restricted to the cecum in the porcupine, whereas the beaver uses both its cecum and the proximal colon [20]. Both animals feed on tree

Table 7. Average percent abundances of bacterial genus’ contributing beta-glucosidase (K05349) in the Arctic wolf, coyote, beaver and porcupine microbiomes as analyzed by HUMAnN2. Average percent abundances were obtained by calculating the percentage of each organism in each run (relative abundance divided by total abundance) and then taking the average of these means. Each sample was sequenced in biological triplicate times, except the porcupine which was sequenced in biological quadruplicate.

| K05349: Beta-glucosidase | Arctic Wolf | Coyote | Beaver | Porcupine |
|--------------------------|-------------|--------|--------|-----------|
| Bacteroides | 42.66% | 47.89% | 41.48% | 30.55% |
| Blifidobacterium | 8.05% | 5.60% | 8.72% | 11.54% |
| Clostridium | 5.84% | 4.85% | 11.96% | 8.55% |
| Parabacteroides | 6.86% | 5.08% | 5.80% | 6.29% |
| Butyribrio | 5.72% | 3.27% | 5.74% | 7.35% |
| Ruminococcus | 3.02% | 3.04% | 3.44% | 6.54% |
| Pedobacter | 4.59% | 6.03% | 1.59% | 2.01% |
| Pseudomonas | 3.39% | 3.83% | 2.07% | 2.98% |
| Olsenella | 2.31% | 2.70% | 2.62% | 4.34% |
| Alicyclobacillus | 2.79% | 2.85% | 2.12% | 3.06% |
| Lactobacillus | 4.20% | 2.13% | 1.98% | 2.75% |
| Eubacterium | 1.70% | 1.73% | 1.97% | 2.96% |
| Cellulophaga | 1.77% | 1.87% | 1.92% | 1.98% |
| Tolumonas | 1.56% | 1.20% | 1.77% | 1.55% |
| Burkholderia | 1.35% | 0.94% | 1.23% | 1.76% |
| Sphingobium | 1.25% | 1.20% | 1.21% | 1.15% |
| Slackia | 0.71% | 0.93% | 1.46% | 1.25% |
| Variovorax | 0.75% | 1.07% | 0.62% | 0.72% |
| Enterococcus | 0.54% | 0.38% | 0.26% | 0.41% |
| Terriglobus | 0.38% | 0.30% | 0.39% | 0.42% |
| Rhodopseudomonas | 0.18% | 0.38% | 0.25% | 0.24% |
| Rhodospirillum | 0.26% | 0.27% | 0.13% | 0.16% |
| Lactococcus | 0.06% | 0.05% | 0.06% | 0.06% |
| Klebsiella | 0.00% | 0.00% | 0.03% | 0.00% |

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Table 8. Average percent abundances of bacterial genus’ contributing to endoglucanase (K01179) in the Arctic Wolf, Coyote, Beaver and Porcupine microbiomes as analyzed by HUMAnN2. Average percent abundances were obtained by calculating the percentage of each organism in each run (relative abundance divided by total abundance) and then taking the average of these means. Each sample was sequenced in biological triplicate.

| K01179: Endoglucanase | Arctic Wolf | Coyote | Beaver | Porcupine |
|------------------------|-------------|--------|--------|-----------|
| Clostridium | 22.95% | 48.66% | 25.83% | 42.82% |
| Eubacterium | 11.51% | 23.52% | 10.06% | 24.93% |
| Methanobrevibacter | 17.49% | 1.23% | 19.13% | 2.18% |
| Lactococcus | 4.96% | 3.25% | 8.19% | 13.73% |
| Deinococcus | 9.00% | 4.55% | 10.77% | 3.28% |
| Alicyclobacillus | 4.98% | 3.54% | 6.14% | 6.87% |
| Burkholderia | 8.67% | 2.08% | 5.13% | 0.17% |
| Cellulophaga | 1.47% | 6.11% | 4.14% | 3.56% |
| Klebsiella | 5.23% | 0.17% | 5.59% | 0.15% |
| Pseudomonas | 7.31% | 1.08% | 1.20% | 0.17% |
| Escherichia | 4.97% | 0.22% | 3.25% | 0.43% |

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bark and similar plant material which likely explains certain similarities between their micro-
biomes, notably including high levels of known cellulose-degrading bacteria. Gruninger, et al.
recently sequenced the beaver microbiome from cecum and fecal samples, examining the
microbial differences at the phylum level [10]. They found that the cecum and fecal micro-
biomes were both dominated by Bacteroidetes and Firmicutes. Our study did not sample from
the beaver cecum, but we also found abundant Bacteroidales (class of Bacteroidetes) and Clo-
stridiales (class of Firmicutes) bacteria in the fecal microbiome. Because porcupines and bea-
vers have enlarged cecum’s housing complex microbial communities, we speculate that the
beaver cecum microbiome would also be dominated by Bacteroidetes and Firmicutes. Unfor-
tunately, V1-V3 primers were used to sequence the 16S rRNA in the Gruninger et al. study,
preventing a direct comparison between our datasets.

The functional diversity of the Canidae and Rodentia microbiomes can be attributed to dis-
tinct diets of carnivores and herbivores. Diet, in association with phylogeny, drives the evolu-
tion of mammalian gut microbiota [50] to the point that species-specific microbiomes share
metabolic pathways with other animals that eat similar diets [51]. In this study, analysis of
mammalian microbiomes might be considered biased as we are not sampling wild mammals,
but instead park mammals that are fed a consistent diet based on species-specific nutritional
requirements. The influence of captivity on microbial diversity remains poorly understood
[11, 50, 52]. In studies in which captivity appeared to affect diversity, captive animals were fed
a diet that differed from what they would experience in the wild [50, 53]. In our study, we
observed low deviation between sample replicates (Fig 5) indicating that the microbiomes of
both wild and captive animals are similar and stable (an observation similar to that made by
Song et al. in cohabiting human microbiomes [54–55]).

Beyond taxonomic analysis, we compared the cellulose-degradation potential of the Arctic
wolf, coyote, beaver, and porcupine microbiomes. To do so we measured starch and sucrose
metabolism pathway abundance (which contains the cellulose metabolism pathway) [56]. We
found that the porcupine and beaver microbiomes contained large abundances of cellulose
metabolism genes (as expected) [10, 14, 57, 58], but low abundances of sugar transporter
genes. The opposite result was observed in the Arctic wolf and coyote microbiomes. This result
may be due to the physiological differences between the digestive tracts of herbivores, espe-
cially hind-gut fermenters, and carnivores [59]. The major feature differentiating these two
groups is the cecum, which is located at the start of the large intestine [59]. In comparison to
carnivores, hind-gut fermenters have a working, enlarged cecum which acts as the site of
microbial digestion and fermentation [59]. This means that in hind-gut fermenters, the major-
ity of food passes through the small intestine undigested. Hind-gut fermentation enables the
breakdown of cellulose-rich plant material, but does not support uptake of nutrients, as
reflected in our results (high abundances metabolizing genes, but low abundances of trans-
porters) [59]. For this reason, hind-gut fermenters have evolved a number of behavioral and
physical adaptations, such as eating shed feces, to maximize nutrient absorption [59]. Alterna-
vatively, carnivores rely on the small intestine, which is replete with carbohydrate transporters,
for digestion and absorption of nutrients (as reflected in our results) [59].

Next, we selected three genes involved in cellulose crystal metabolism, di- and tri-saccha-
ride subunit metabolism, and sugar utilization (endoglucanase, beta-glucosidase and cellulose
phosphorylase, respectively). We identified that the porcupine and beaver had significantly
increased abundances of endoglucanase, and cellulobiose phosphorylase (Fig 5). The abundance
of endoglucanase in the porcupine and beaver were corroborated by PICRUSt, HUMANN1,
and HUMANN2 analysis (Figs 5 and 6). HUMANN2 analysis also provided a taxonomic break-
down of organisms, such as Rhodospirillum and Cellulophaga, likely contributing beta-glucosi-
dase and endoglucanase in the porcupine, beaver, Arctic wolf, and coyote microbiomes (Fig
6C). Although *Bacteroides* are abundant in the beta-glucosidase functional assignment for each animal, *Clostridium* are responsible for roughly a two-fold increase of the enzyme in the beaver and porcupine in comparison to the coyote and Arctic wolf. This is to be expected, as *Clostridium* are often noted as important cellulose degraders [57]. For endoglucanase, *Eubacterium* and *Clostridium* play a nearly two-fold greater role in cellulolic breakdown in the herbivores, than in the Arctic wolf and coyote. This is perhaps not surprising, as the organisms that metabolize cellulose (like *Clostridium* spp.) are more abundant, thus there is a larger abundance of reads matching cellulose degradation.

Most of the enzymes needed to degrade complex plant polysaccharides are not present in mammalian genomes, resulting in a mutual dependence between the mammalian host and gut microbiota [58]. Zhu et al. investigated the bacterial diversity of the giant panda (*Ailuropoda melanoleuca*) which possesses a typical carnivore gastrointestinal tract, but which also consumes roughly 12.5 kg of bamboo each day [60]. Using 16S rRNA sequencing and gene function classification they identified cellulose-metabolizing bacteria in the panda microbiome. Separate groups have identified the cellulolytic potential of other microbiomes such as beavers [10], ruminants [14], and termites [61]. In our datasets, the porcupine and beaver had statistically higher abundances of endoglucanases and celluliose phosphorylases than carnivores. Thus, mining animal gut microbiomes could provide a potentially rich source of novel microbial genes that could be leveraged for bioengineering applications.

Metagenomic libraries derived from hind-gut fermenter fecal samples could accelerate the discovery of novel cellulolytic enzymes using an approach known as synthetic metagenomics [62–63]. This technique chemically synthesizes selected genes of interest identified from functional metagenomic screens [63]. Synthetic metagenomics enables the identification and synthesis of thousands of novel enzyme sequences from complex environmental isolates. Combining traditional metagenomic library analysis with new synthetic metagenomics approaches will undoubtedly accelerate efforts to “mine the microbiome” of mammals of interest.

**Supporting information**

S1 Fig. Alpha rarefaction curve of the 21 mammal microbiomes sequenced by 16S rRNA sequencing. All animals sequenced in the initial microbiome survey are included above. Each line represents the function of the observed number of OTUs over the sequences per sample. (TIF)

S2 Fig. Alpha rarefaction curve of the replicate mammal microbiomes sequenced by 16S rRNA sequencing. The arctic wolf, beaver, coyote and porcupine, all which were sequenced in triplicate, are represented above. Each line represents the function of the observed number of OTUs over the sequences per sample. (TIF)

S1 Table. 16S taxonomy for all 21 mammals at Shubenacadie Wildlife Park. (XLSX)

S2 Table. 16S taxonomy for the 4 mammals (Arctic Wolf, Coyote, Beaver, Porcupine) sequenced in triplicate at Shubenacadie Wildlife Park. (XLSX)

S3 Table. MetaPhlAn taxonomic assignment data from metagenomic sequencing for the Arctic Wolf, Coyote, Beaver and Porcupine. (XLSX)
S4 Table. HUMAnN functional assignment data from metagenomic sequencing for the Arctic Wolf, Coyote, Beaver and Porcupine. (XLSX)

S5 Table. HUMAnN2 functional gene family assignment data from metagenomic sequencing for the Arctic Wolf, Coyote, Beaver and Porcupine. (ZIP)

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