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THE CECAL MICROBIAL COMMUNITY'S RESPONSE TO HIBERNATION OF THIRTEEN-LINED GROUND SQUIRRELS

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THE CECAL MICROBIAL COMMUNITY'S RESPONSE TO HIBERNATION OF THIRTEEN-LINED GROUND SQUIRRELS

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We recommend acceptance of this thesis in partial fulfillment of the candidate's requirements for the degree of Masters of Science in Microbiology

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ABSTRACT

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The presence of microbial communities within the gastrointestinal tract is extremely important. Intestinal associated microbial populations are established immediately after birth and the population is maintained by diet. Hibernation is an adaptation that some animals use, during which an animal does not consume additional food. Due to this change in diet, a significant change in the intestinal microbiota is expected. Cecal material was collected from thirteen-lined ground squirrels at various stages relative to hibernation: pre-hibernation, inter-bout arousal, two hours post arousal, twenty-four hours post arousal, and seven days post arousal. Each cecal sample was used for community analysis via DNA extraction and sequencing of 16S rRNA genes and used to determine abundance via flow cytometry. Community analysis identified a progression where microbial communities prior to hibernation were dominated by phylum Firmicutes but during hibernation the community was dominated by Bacteroidetes. Each progressive stage post arousal from hibernation showed an increase in Firmicutes relative to Bacteroidetes culminating in a similar community structure at seven days post arousal to that seen pre-hibernation. Abundance followed a cycle starting with high abundance pre-hibernation followed by a significant reduction during inter-bout arousal. Abundance gradually restored at each stage post-arousal culminating in equivalent abundance to pre-hibernation.

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INTRODUCTION

While the internal tissues of an animal are generally free from microbes, this is not the case for the entire body. There is a substantial relationship between microbes that naturally reside inside or on an animal, referred to as normal flora, and the animal itself (1). Normal flora is found in the oral cavity, in the gastrointestinal tract, and on the skin. While the relationship between the host and its microbial inhabitants is far from completely understood, research has shown that normal flora has a significant impact on the health of the host.

Normal flora is beneficial to the host, as it acts as a physical barrier to prevent attachment of potential pathogens (1). This barrier effect applies both to transient pathogens as well as keeping resident opportunistic pathogens in check. Disturbing the equilibrium established by normal flora, as with the use of antibiotics, can result in a significantly higher risk of infection by an opportunistic pathogen (2). While this behavior as a barrier is certainly important, normal flora performs additional functions within the host. These resident microbes also metabolize dietary residues and other compounds that the host is incapable of digesting (3). The result is an ability to degrade a far wider selection of compounds than the host can alone. In some instances these additional pathways can satisfy up to 20% of a host animal's energy requirements (4). The additional metabolic pathways are largely fermentations, so often the organic end product is something that the host can use, typically short chain fatty acids (2,5). Research has indicated that an absence of gut normal flora during development leads to

reduced vascularity, digestive enzyme activity, muscle wall thickness, cytokine production and serum immunoglobulin levels. The introduction of a single bacterial species into the gastrointestinal tract, however, will reverse these trends largely by the restored production of these short chain fatty acids that alter the development of gut epithelium (6,7,8). Correlation has also begun to emerge between gut normal flora and certain diseases (2). This interplay has been extensively demonstrated in the manifestation of inflammatory bowel disease (9), but links have also been made to obesity (2), type 1 diabetes (10), Crohn's disease (11), as well as colorectal cancers (2,12). A variety of factors can lead to these diseases, ranging from host dietary choices resulting in the production of harmful metabolites by microbial flora to hyper-reactivity of the host immune system to normal flora antigens (2,10,11).

In general, gut normal flora has been shown to have extensive interplay with the host immune system. This can be attributed to the significant involvement of the immune system in gut epithelial tissues, because of the large number of antigens present within the gut from both diet and resident microbes. In conjunction with the high levels of antigens present in the gastrointestinal tract, there is also a very high concentration of immune system cells in the intestinal epithelium (1,2). Studies using animals born and raised in a sterile environment have shown that normal flora is essential for the development of proper immune system function as a result of microbes interacting with these immune system cells (6,13). If development reaches a certain point and microflora has not been introduced, the immune system cannot fully mature (7). A major effect of this diminished development is the systemic immune response to antigens that would normally be ignored.

Given the importance of gut normal flora on health, the effect of diet and lifestyle on its composition should be considered. Initially, a fetus' gut is sterile at birth, but colonization quickly begins and is influenced by a variety of factors (1). These factors include diet (2), mode of delivery at birth (14), hygiene levels (15), and the use of any medications (1). Although a certain number of early colonizers are eventually lost, over time a community is established based on this early colonization and diet. As a result, radical changes in diet as well as fasting events could have a large effect on gut normal flora. One such fasting event is hibernation, which has been used as a model for starvation (5).

Hibernation is a method that some mammals have developed as a response to the unfavorable conditions during winter. The animal enters a state of torpor in which body temperature is only slightly above ambient temperatures as a result of the minimization of energy expenditure (14). While some mammals have evolved to rouse at intervals to either consume cached food or forage for available food, many species consume no food over the duration of hibernation whether they have inter-bout arousals or not. One such species is *Spermophilus tridecemlineatus*, or the thirteen-lined ground squirrel as it is commonly known (14,16). Due to this fasting caused by hibernation, the expectation would be that gut normal flora of the squirrel is altered. A previous study investigated the effect of hibernation on cecal microflora in thirteen-lined ground squirrels by examining changes in the microbial community between hibernating and active animals using direct microscopic examination and anaerobic culturing techniques (17). Due to the age of the study no phylogenetic analysis was conducted as the technologies and infrastructure for that type of data generation and analysis did not exist. Other studies of this nature have

been done on hibernating animals, such as the Syrian hamsters (18), leopard frogs (19), European squirrels (20), and marmots (16); however, with the exception of work on Syrian hamsters, these studies have focused largely on the ability of pathogens to survive the hibernation event rather than on the effect of hibernation on the gut flora. The data in these studies were largely composed of general classifications for groups of microbes rather than identifying any specific species. As such, a modern study investigating quantitatively how hibernation impacts abundance of microflora as well as phylogenetic analysis into the diversity and how diversity changes during hibernation is of interest.

Modern high throughput sequencing techniques have been used extensively in recent years for phylogenetic analysis of many different microbial communities, including that of gut normal flora. The appeal of these high throughput sequencing processes is the speed and lower cost as well as the volume of data that can be generated. A study of particular note is the Human Microbiome Project (21), which is aimed at identifying all of the microbial species that are associated with humans. Among the myriad of other investigations into microbial normal flora communities, some studies have been done investigating the effect of hibernation on microbial communities within a host organism. Phylogenetic analyses of microbial communities within the cecal material of arctic ground squirrels (22), and thirteen-lined ground squirrels (23) have been performed. These studies predominantly investigated changes within the torpor state of hibernation as compared to pre-hibernation and compared to a timepoint two weeks or more after the end of hibernation. Changes in microbial community structure were observed between early hibernation and late hibernation, however no study has been conducted investigating multiple timepoints following the end of hibernation to elucidate

the rate with which community restructuring occurs. Additionally, the study involving thirteen-lined ground squirrels focused predominantly on community diversity via phylogenetic analysis without investigating directly observed abundance of the microbial communities throughout hibernation. These studies both identified a cyclical pattern with respect to the relative abundance of cecal microbiota over the course of hibernation. Prior to hibernation cecal microbial communities were dominated by members of the phylum Firmicutes with members of the phylum Bacteroidetes being the next highest in relative abundance but to a significantly lower extent. During torpor and during inter-bout arousals during hibernation this relative abundance shifted to a structure dominated by Bacteroidetes and with a much decreased Firmicutes presence. Two weeks post arousal from hibernation the community was observed to have returned to similar characteristics to that of the pre-hibernation state.

In our study, we collected the cecal contents from thirteen-lined ground squirrels at multiple timepoints relating to hibernation. The cecum, which is responsible for removing water and salts during digestion as well as beginning feces formation, was chosen due to the fact that previous hibernation studies into microflora have been performed on cecal contents. The timepoints were pre-hibernation, during an inter-bout arousal event, 2 hours post arousal, 24 hours post arousal, and 7 days post arousal. These timepoints allowed an investigation into the kinetics of how the microbial normal flora community develops immediately following hibernation rather than weeks later. Using the cecal material, we extracted DNA and had Ion Torrent sequencing performed. Additionally, we fixed and stained a portion of the cecal material to count the microbial population using flow cytometry. The data from phylogenetic analysis of the DNA

sequences across multiple timepoints following arousal, taken with the abundance data, allowed us to provide additional perspective on the cyclical pattern with which cecal microbial communities respond to hibernation that had been identified in previous studies.

MATERIALS AND METHODS

Animals

Ground squirrels were born in captivity and housed at the University of Wisconsin-La Crosse following protocols approved by the Institutional Animal Care and Use Committee (IACUC). Wild caught squirrels have regularly been introduced into the colony. Non-hibernating animals were housed individually in rat caging on a Wisconsin photoperiod. In the fall, when an animal's body temperature dropped to 25°C (ambient), animals were placed in 8" x 8" plastic containers with bedding and moved into a 4°C hibernaculum. Animals were checked daily for arousal, and if they were alert for 2 consecutive days they were moved out of the hibernaculum. Animals were euthanized by isoflurane anesthesia followed by decapitation and then dissection and organ removal. Four dissection events occurred between October 2011 and February 2012 resulting in a total of 30 samples collected (Table 1). The squirrels dissected were mixed in gender and in age, and included some wild caught animals. Six total animals were dissected for each timepoint.

TABLE 1 Dates of thirteen-lined ground squirrel dissection events

Date	Stage	Label
10/13/2011	Pre-hibernation	Pre1 through Pre6
1/5/2012	Inter-bout arousal	IB1 through IB6
2/16/2012	2 hours post arousal	PA2-1 through PA2-6
2/16/2012	24 hours post arousal	PA24-1 through PA24-3
2/22/2012	24 hours post arousal	PA24-4 through PA24-6
2/22/2012	7 days post arousal	PA7-1 through PA7-6

Dissection

At each dissection, the cecum was removed and placed into a pre-weighed conical tube, which was immediately chilled on ice for temporary storage and transportation. The tube containing the cecum was weighed, and the total cecal mass calculated for each sample. Each cecal sample, including cecal wall and contents, was split in two using a sterile scalpel so that one portion could be directly frozen at -20°C and the other portion could be fixed with paraformaldehyde (Table 2). The sample separated for fixation was weighed and this weight subtracted from the original cecal weight to determine the amount that remained in the frozen sample.

Fixed Sample Preparation

Fresh 4% paraformaldehyde was prepared on the day of each dissection following the protocol from the Chromaffin Cell and Hypertension Research group at UCSD (http://hypertension.ucsd.edu/). After the cecal samples had been split, 2-3 ml of paraformaldehyde was added to one portion of each sample. The samples were then homogenized in the paraformaldehyde and fixed at 4°C for 24 hours. Homogenization was performed using a combination of sterile applicator sticks and sterile rod attachments for a Norpro (Everett, WA) handheld mini-mixer. Following fixation, the samples were centrifuged in a bucket rotor centrifuge at 200 RCF and room temperature for 20 minutes and the supernatant was decanted. Two wash steps were performed on each sample using 3 ml of 4°C filtered-sterilized phosphate buffered saline (PBS) and centrifuged again. After the final decanting of the wash PBS, 2.5 ml of filtered-sterilized PBS and 2.5 ml of 70% ethanol were added for long term storage at -20°C.

DNA Extraction

DNA was extracted from the frozen portion of each cecal sample for sequencing. Samples were first thawed and then homogenized using a combination of sterile applicator sticks and sterile rod attachments for a Norpro (Everett, WA) handheld mini-mixer. Once a uniform consistency was achieved, 100 mg of the cecal contents was removed to be used with a fecal DNA extraction kit (Zymo Research, Irvine, CA) following the included protocol. The extracted DNA samples were run on a 1.0% agarose gel to assess DNA quality. Each satisfactory DNA sample was diluted 1:100 and Hoechst dye 33342 added at a concentration of 40 µg/ml to determine the DNA concentration using a fluorescent bioanalyzer microtiter plate reader (BioTech, Winooski, VT). Based on the concentration of DNA in each sample, dilutions were performed to obtain 20 µl of each sample at 20 ng/µl final DNA concentration. These samples were then shipped overnight to Molecular Research DNA Laboratory in Stillwater, TX for Ion Torrent DNA sequencing services.

Flow Cytometry

The samples fixed in paraformaldehyde were diluted $1/10^5$ to achieve a concentration that could be more accurately counted on a flow cytometer. The samples were then stained using 10 µl of a 1 to 100 dilution of 0.025% acridine orange stain into 990 µl of the diluted paraformaldehyde fixed cells. Cells were stained for 24 hours (Panel discussion at ISME14, Copenhagen, Denmark, 19 to 24 August 2012) prior to counting on a BD Accuri C6 Plus flow cytometer (Franklin Lanes, NJ). Gating for the flow cytometer method was established using log phase, acridine orange-stained *Escherichia coli* and pure filter-sterilized PBS to determine an approximate lower limit on cell size

TABLE 2 Thirteen-lined ground squirrel body and cecal mass

Sample ID[a]	Animal weight (g)	Cecum weight (g)	Cecum weight (% of total weight)	Frozen weight[b] (g)	Fixed weight[b] (g)
Pre1	229	4.96	2.17	2.17	2.79
Pre2	221	3.11	1.41	1.40	1.71
Pre3	202	4.82	2.39	2.32	2.50
Pre4	220	5.05	2.30	3.37	1.68
Pre5	262	3.50	1.34	1.63	1.87
Pre6	196	3.35	1.71	1.51	1.78
IB1	221	1.33	0.60	0.60	0.73
IB2	205	1.51	0.74	0.75	0.76
IB3	196	1.20	0.61	0.46	0.74
IB4	160	2.43	1.52	1.28	1.15
IB5	166	1.60	0.96	0.87	0.73
IB6	202	1.65	0.82	0.68	0.93
PA2-1	183	1.72	0.94	0.94	0.78
PA2-2	149	1.11	0.74	0.56	0.55
PA2-3	168	1.07	0.64	0.65	0.42
PA2-4	154	1.20	0.78	0.78	0.42
PA2-5	133	1.66	1.25	0.51	1.15
PA2-6	181	1.63	0.90	0.65	0.98
PA24-1	188	4.82	2.56	1.74	3.08
PA24-2	134	2.32	1.73	1.34	0.98
PA24-3	116	2.40	2.07	0.93	1.47
PA24-4	169	2.61	1.54	1.33	1.28
PA24-5	197	2.78	1.41	1.54	1.24
PA24-6	138	3.21	2.33	1.64	1.57
PA7-1	163	2.27	1.39	1.18	1.09
PA7-2	178	2.44	1.37	1.41	1.03
PA7-3	183	2.17	1.19	0.88	1.29
PA7-4	133	4.30	3.23	1.79	2.51
PA7-5	151	4.01	2.66	1.43	2.58
PA7-6	106	3.07	2.90	1.24	1.83

[a]Sample ID prefixes refer to hibernation stages. Pre for pre-hibernation, IB for inter-bout arousal, PA2 for 2 hours post arousal, PA24 for 24 hours post arousal, and PA7 for 7 days post arousal.

[b]Frozen and fixed weights refer to quantities of cecal material after division of the original samples.

and background noise, respectively. Counting was performed on a volume basis, with a sample size of 34 µl.

DNA Sequencing

Sequencing of the extracted DNA was performed by Molecular Research DNA Laboratory (MR DNA). The 16S rRNA gene sequences with an average Phred quality score of 25 (99.5% confidence in base reads) across the sequence were processed using a proprietary analysis pipeline (www.mrdnalab.com). Sequences had barcodes and primers removed, followed by the removal of sequences below 200 base pairs in length. Sequences with ambiguous base calls and sequences with homopolymer runs more than 6 bp long were also removed. The remaining sequences were then denoised and had chimeras removed. Operational taxonomic units (OTUs) were defined after removal of singleton sequences, with clustering at 97% similarity. The OTUs were then taxonomically classified using BLASTn against a curated GreenGenes database (http://greengenes.lbl.gov/) and compiled into each taxonomic level in files with count data. The count data files contained actual number of sequence reads of each OTU present in each sample.

Sequence Data Analysis

The OTU count data returned from Molecular Research DNA Laboratory was used to create two files, one containing OTU and sample read data and the other containing OTU and taxonomic data. An annotation file was then created with our metadata. The raw read data was used to generate heatmaps and alpha diversity using the phyloseq (24) package in R and a rarefaction curve was produced using the VEGAN (25) package in R. Alpha diversity measures used were Chao1 diversity index, Simpson

diversity index, and Shannon diversity index. The equation used for Chao1 was (observed + singles*(singles-1) / (2*(doubles+1)). The equation used for Simpson diversity index was (1 – ((Σ n(n-1))/(N(N-1))) where n is the number of reads for a specific OTU and N is the total reads. The equation used for Shannon diversity index was ($-\Sigma p_i \ln(p_i)$) where p_i is the proportion of total reads assigned to the same OTU. Raw read data was converted to relative abundance for use in preparing stacked bar plots with phyloseq. Raw data were transformed using an rlog variance stabilizing transformation which was then used for principal component analysis, both performed using the DESeq2 (26) package in R.

RESULTS

Flow Cytometry Cell Counts of Cecal Contents

The bacterial counts per mg of cecal contents for all of the samples counted ranged from as low as 310 up to 6860 (Fig. 1A-1E). The bacterial counts per mg of cecal contents varied to a large extent within each sample timepoint, however a significant trend was still observed. From pre-hibernation into inter-bout arousal a greater than 75% reduction in bacterial abundance was observed. After this substantial reduction, however, a steady increase in abundance was seen progressing through the post arousal stages, culminating at 7 days post arousal which had abundance levels equivalent to those at the pre-hibernation timepoint (Fig. 1F).

The abundance counts for pre-hibernation and 7 days post arousal were not significantly different from each other ($p > 0.5$) while also being significantly different from the inter-bout arousal and 2 hours post arousal sample timepoints ($p < 0.05$). Additionally, the bacterial abundance of inter-bout arousal samples was significantly different from all but the 2 hours post arousal samples ($p < 0.05$). Due to the high variability observed, the samples from 24 hours post arousal only significantly differed from the inter-bout arousal samples ($p < 0.05$).

Ion Torrent 16S rRNA sequencing

Ion Torrent sequencing of 16S rRNA genes from extracted DNA obtained from the cecal material resulted in total sequence reads ranging from 9417 to 29251, and total OTUs ranging from 2813 to 5793 per sample. One sample, PA2-4, returned only a single

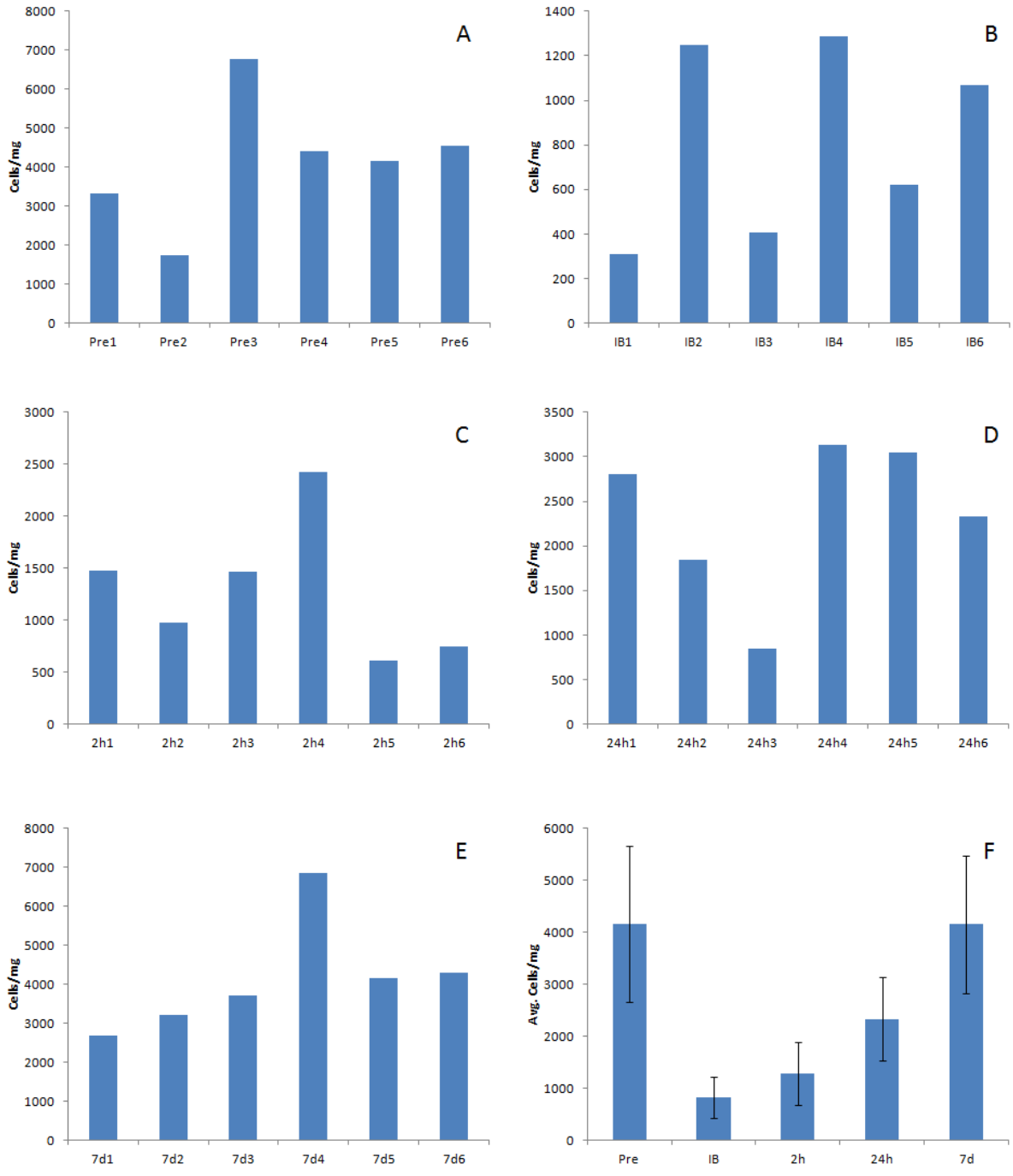

FIG. 1. Bacterial cell counts from cecal contents of thirteen lined ground squirrels measured using flow cytometry. Cell counts were normalized to mass (mg) of fixed cecal material. Ceca (n=6) were harvested prior to hibernation (A), during inter-bout arousal (B), and at 2 hours (C), 24 hours (D), and 7 days (E) after hibernation. Panel F compares the mean counts from each timepoint.

read and OTU (Table 3). It was assumed that this was the result of either an error in sample handling or preparation, or due to an error in the sequencing process. This sample was removed before further data processing was performed.

Each category of samples, pre-hibernation, inter-bout arousal, and the three post-hibernation times, had a sample randomly chosen to be sequenced in duplicate (Table 3). The duplicate samples were compared to determine if the sequencing process or other handling introduced any bias or deviation into the data. An order level comparison of the sequence data provided very similar community profiles between each duplicate set (Fig. 2). Given the similar read numbers and community composition of the duplicate samples, the B samples from each duplicate set were removed before further data processing was performed. The duplicate samples were neither combined nor averaged so that each sample was treated identically, since the remaining samples did not have a duplicate to consider.

The total reads and OTU data obtained from each sample were used to generate rarefaction curves (Fig. 3). The rarefaction curves have not completely leveled off, indicating that additional OTUs could be obtained from additional sequencing. However, most of the samples appear to have passed the inflection point on the curve indicating that the majority of OTUs have likely been obtained. Additionally, the community abundance distribution of the duplicate samples yielded similar profiles (Fig. 2). Since sets of two independently sequenced duplicate samples resulted in similar relative abundances of community constituents, it may be unlikely that additional sequencing would significantly alter these profiles.

TABLE 3. Total OTU[a] and total reads[a] from 16S rRNA sequencing of the cecal contents harvested from thirteen-lined ground squirrels before, during, and after hibernation

Sample ID[b]	Total OTUs	Total Reads
Pre1	3713	11827
Pre2	3312	9417
Pre3	4265	17672
Pre4	4539	15976
Pre5A[c]	4225	13962
Pre5B[c]	4601	15837
Pre6	3754	18340
IB1	4780	19590
IB2	4405	25355
IB3	4173	21857
IB4	4128	14995
IB5A[c]	3223	10154
IB5B[c]	4074	15000
IB6	5188	23986
PA2-1	5108	24271
PA2-2	4687	19282
PA2-3A[c]	5339	29251
PA2-3B[c]	4549	23372
PA2-4	1	1
PA2-5	5148	20070
PA2-6	5073	19966
PA24-1A[c]	4399	13908
PA24-1B[c]	4014	12479
PA24-2	4950	16366
PA24-3	5215	18076
PA24-4	5437	17375
PA24-5	5793	24100
PA24-6	5333	21112
PA7-1A[c]	3374	18332
PA7-1B[c]	3404	18768
PA7-2	4822	16926
PA7-3	2813	14297
PA7-4	4758	21666
PA7-5	5090	18358
PA7-6	5016	15561

[a]Totals based on data available in Appendix 1.

[b]Sample ID prefixes refer to hibernation stages. Pre for pre-hibernation, IB for inter-bout arousal, PA2 for 2 hours post arousal, PA24 for 24 hours post arousal, and PA7 for 7 days post arousal.

[c]Denotes samples run in duplicate during sequencing.

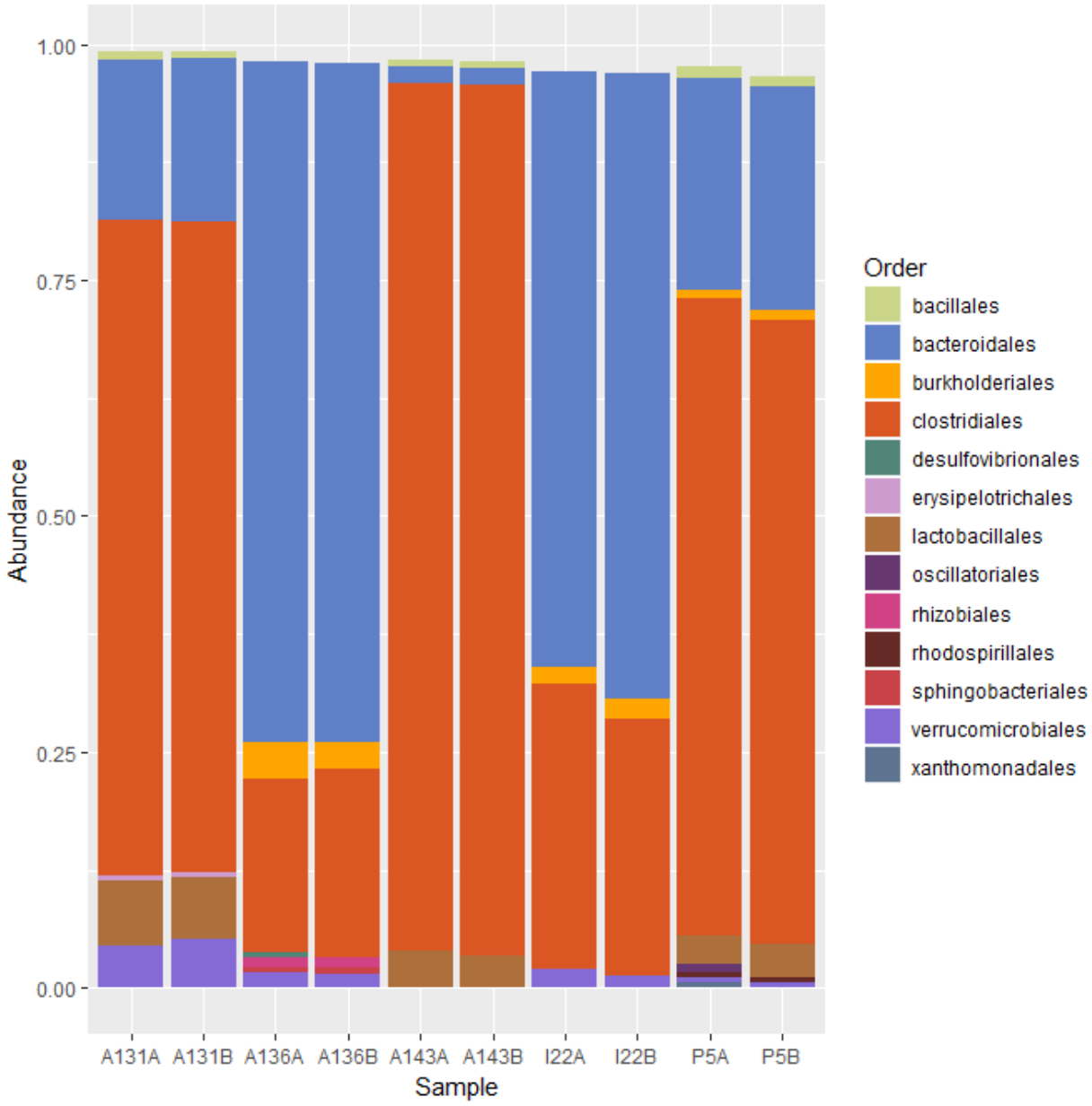

FIG. 2. Stacked bar chart of relative abundance showing order level comparison of duplicate samples from bacterial communities. Sequences were obtained from DNA extracted from the cecal contents of thirteen-lined ground squirrels at timepoints related to hibernation. Sample states were pre-hibernation (P5A, P5B), inter-bout arousal (I22A, I22B), 2 hours post arousal (A136A, A136B), 24 hours post arousal (A131A, A131B), and 7 days post arousal (A143A, A143B). Duplicate samples were run to ensure reproducibility of sequencing process. OTUs making up less than 0.5% of the total sequences in a given sample are not shown.

Alpha diversity measures of OTU reads indicated a high degree of diversity within each cecal sample. Chao1 index scores ranged from just below 5000 up to slightly above 10000 (Fig. 4). Comparing the Chao1 scores to total OTU numbers again indicated that some additional OTUs likely would have been obtained through additional sequencing, similar to the rarefaction curves. Simpson diversity index values were at or above 0.97, which means a very high degree of diversity as this value approaches 1 (Fig. 5). Additionally, Shannon diversity index values also indicate a high degree of diversity, with values ranging from 6.2 up to 7.6 (Fig. 6). The high Shannon index values also indicate that community members should be more evenly represented, and that one OTU is not completely dominating any sample.

Phylum level analysis of the relative abundance of OTUs present in a given sample showed Firmicutes and Bacteroidetes present in all samples and also comprising the majority of each community sampled (Fig. 7). Additionally, Proteobacteria, Tenericutes, and Verrucomicrobia were present in many of the samples but to a lesser extent. Two samples, A5 and B3 from pre-hibernation and inter-bout arousal respectively, reported a small number of sequences from the phylum Cyanobacteria, the majority of which were from the order Oscillatoriales. These sequences may have been the result of plant matter still present in the cecum. Actinobacteria were present in only three samples at a level above 0.5%, samples B2 and B6 from inter-bout arousal and sample C4 from 2 hours post arousal. All of the Actinobacteria sequences were found within Actinomycetales or Coriobacteriales. Relative abundance of Firmicutes was high prior to hibernation then decreased during hibernation as shown in the inter-bout arousal samples. After hibernation ended, relative abundance of Firmicutes increased with each

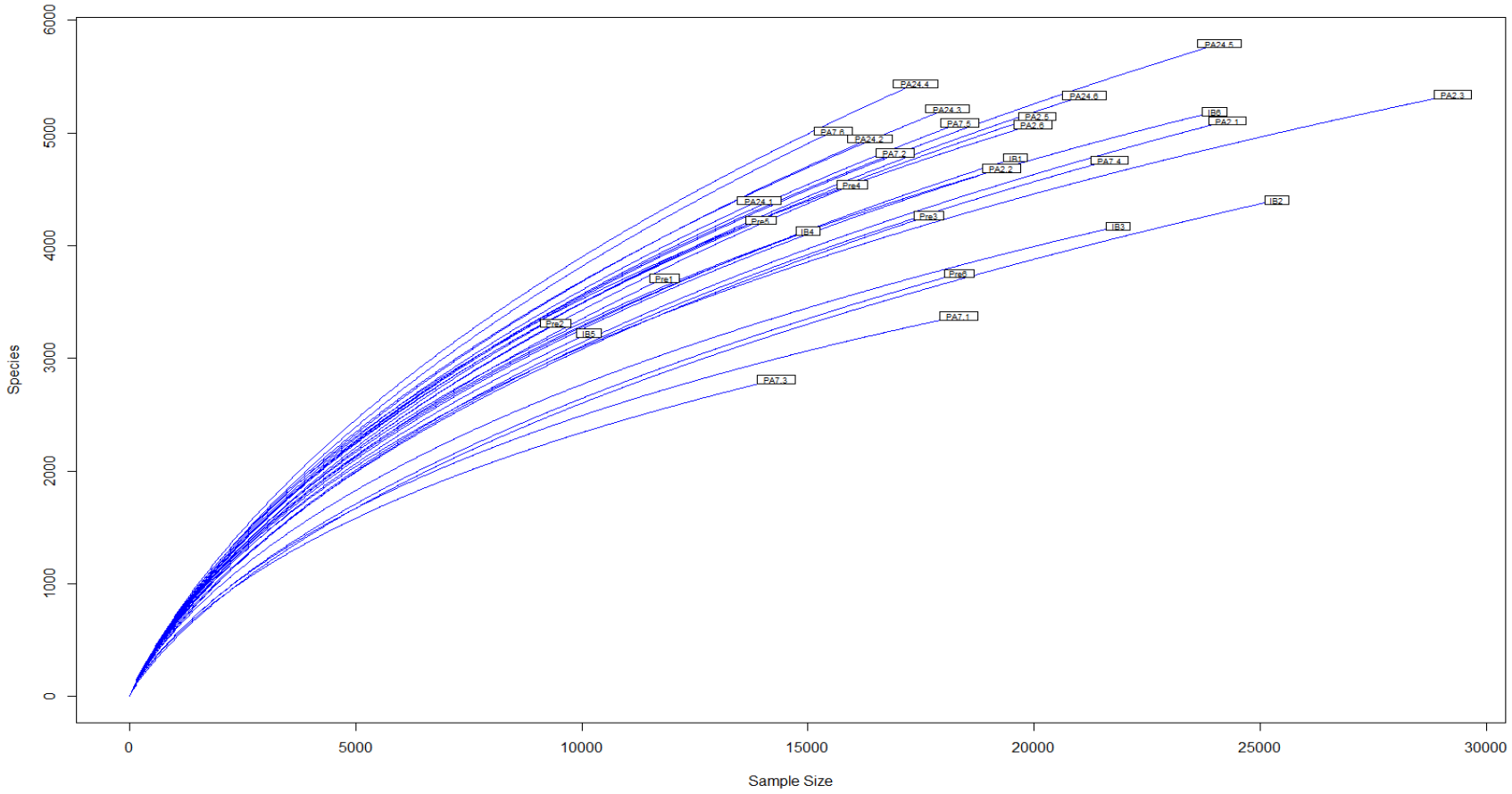

FIG. 3. Rarefaction curves for DNA sequence reads (sample size) and OTU data generated from bacterial 16S rRNA sequences extracted from the cecal contents of thirteen-lined ground squirrels at timepoints related to hibernation. Sample states were pre-hibernation (Pre1 - Pre6), inter-bout arousal (IB1 - IB6), 2 hours post arousal (PA2.1 - PA2.5), 24 hours post arousal (PA24.1 - PA24.6), and 7 days post arousal (PA7.1 - PA7.6).

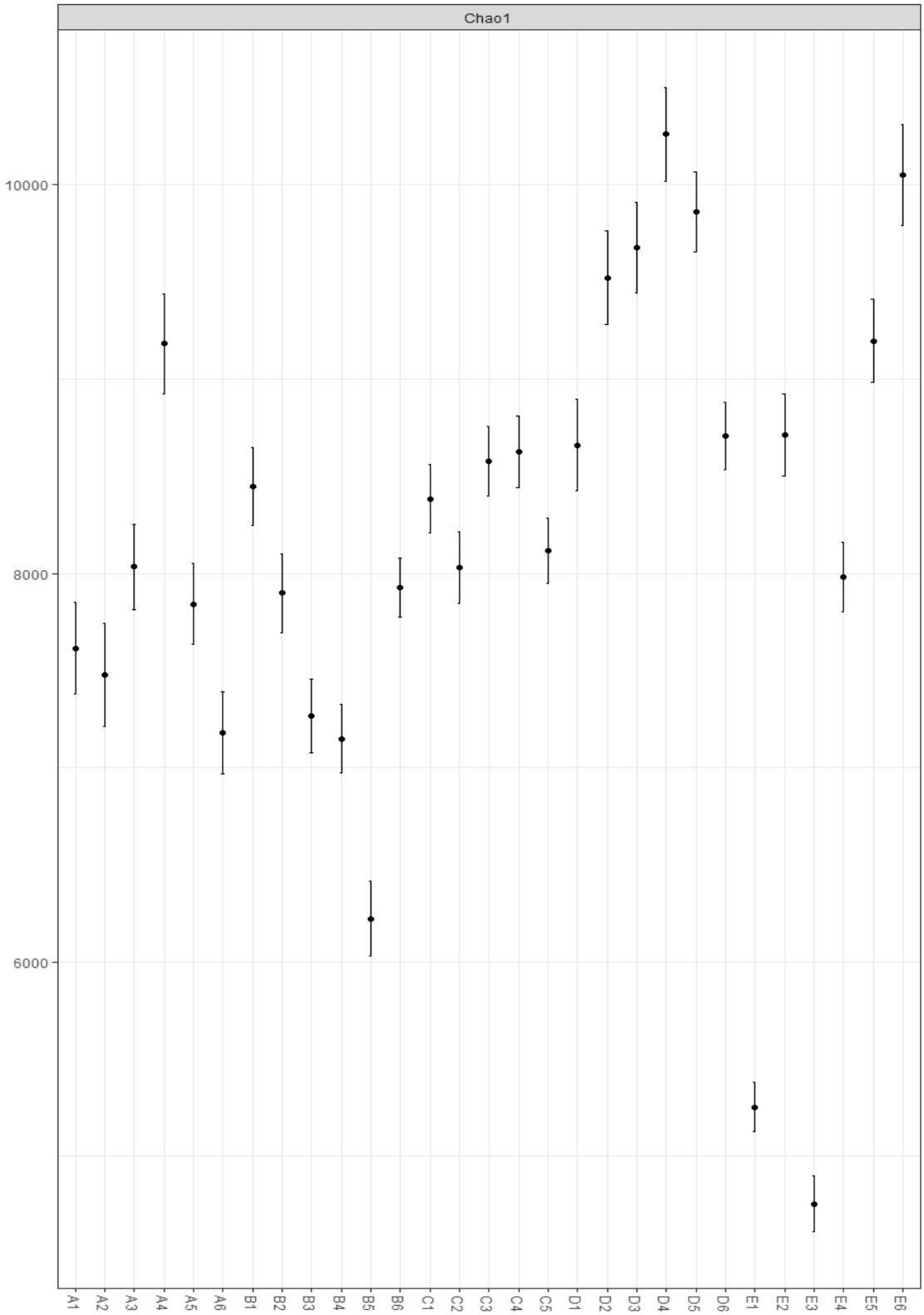

Fig. 4. Chao1 alpha diversity index values for OTUs present in the cecal contents of thirteen-lined ground squirrels prior to hibernation (A1-A6), during inter-bout arousal (B1-B6), and after 2 hours (C1-C5), 24 hours (D1-D6), and 7 days (E1-E6) post arousal.

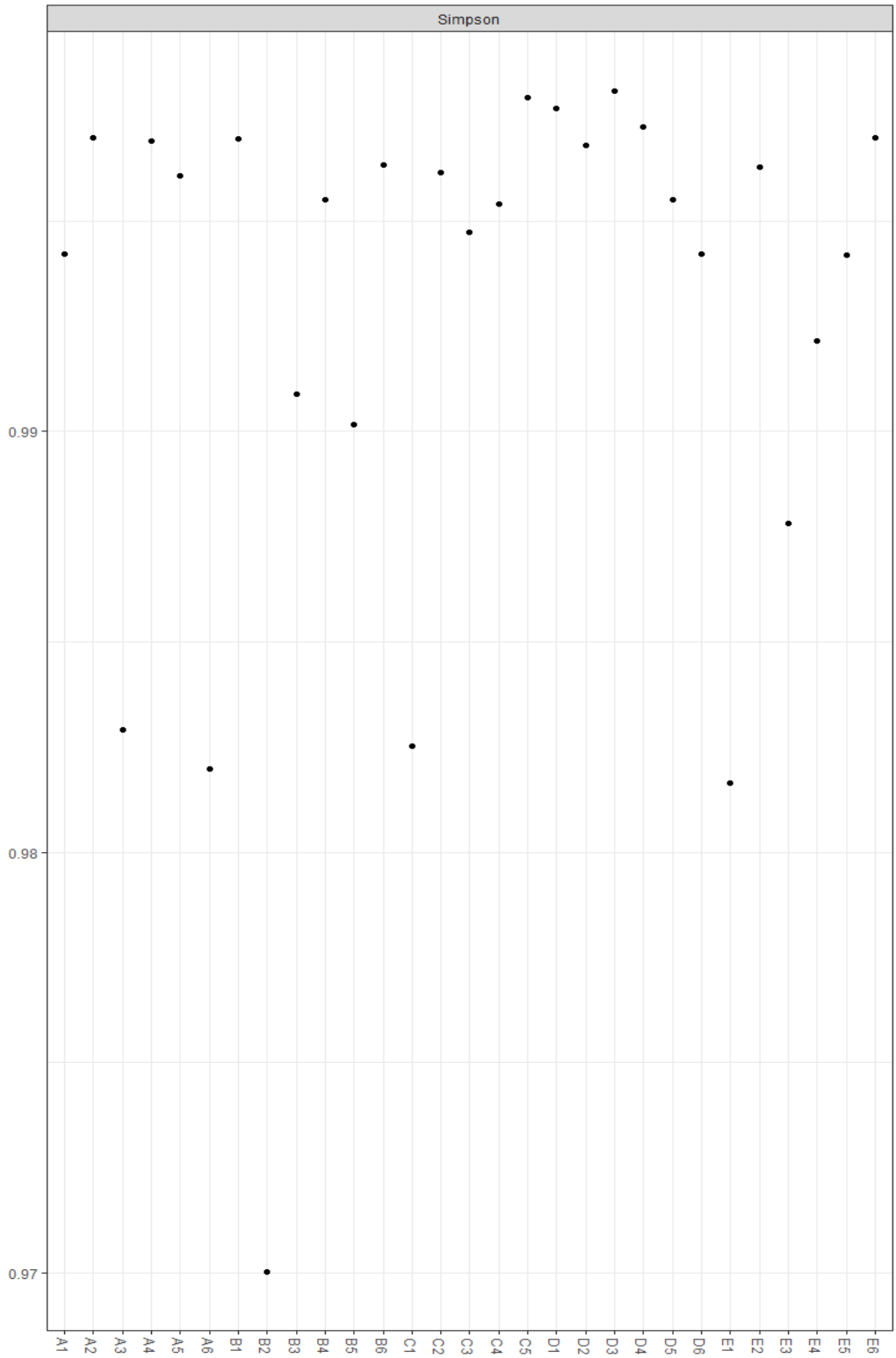

Fig. 5. Simpson diversity index values for OTUs present in the cecal contents of thirteen-lined ground squirrels prior to hibernation (A1-A6), during inter-bout arousal (B1-B6), and after 2 hours (C1-C5), 24 hours (D1-D6), and 7 days (E1-E6) post arousal.

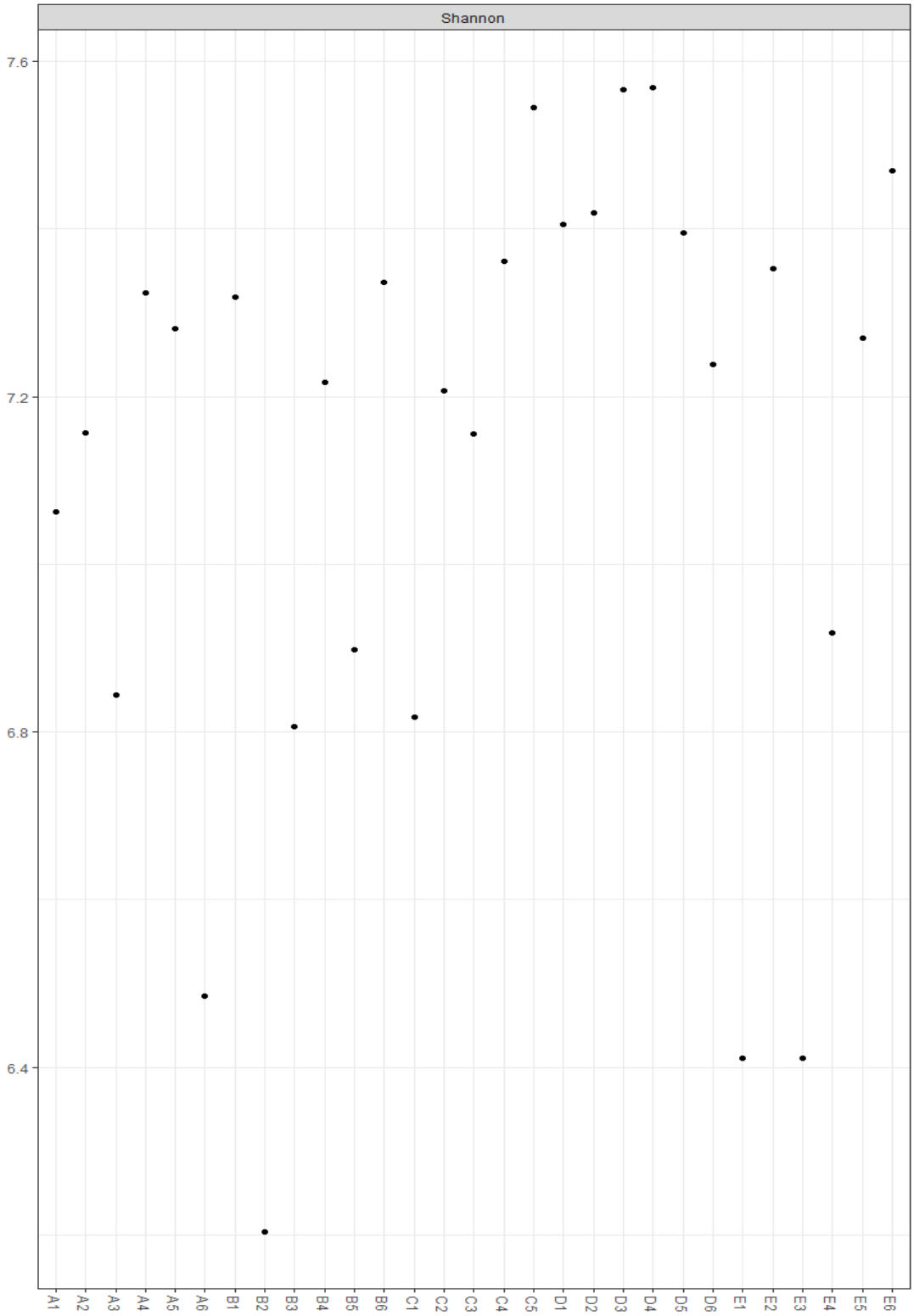

Fig. 6. Shannon diversity index values for OTUs present in the cecal contents of thirteen-lined ground squirrels prior to hibernation (A1-A6), during inter-bout arousal (B1-B6), and after 2 hours (C1-C5), 24 hours (D1-D6), and 7 days (E1-E6) post arousal.

successive sampling, and achieved levels similar to or greater than those observed in pre-hibernation by the 7 day post arousal timepoint samples. Relative abundance of Bacteroidetes showed an inverse relationship to the Firmicutes results, being most abundant during hibernation and diminishing to pre-hibernation levels over the course of post arousal sampling. Verrucomicrobia, except for in sample A6 from pre-hibernation, were at a higher relative abundance during hibernation, and did not diminish to pre-hibernation levels until after 24 hours post arousal.

The relative abundance of OTUs resolved to the order level showed that OTUs within the phylum Firmicutes were comprised almost entirely of Clostridiales and Lactobacillales (Fig. 8). The observed Firmicutes population reduction during hibernation was largely the result of a near disappearance of Lactobacillales. Clostridiales was highly abundant prior to hibernation, but also underwent a decrease during the inter-bout arousal stage though not to the same degree as Lactobacillales. Both Clostridiales and Lactobacillales had returned to pre-hibernation levels by the 24 hours post arousal stage.

A large number of OTUs present from phylum Firmicutes were shared between pre-hibernation samples and the samples from both 24 hours and 7 days post arousal (Fig. 9). The remaining sample groups, inter-bout arousal and 2 hours post arousal, were lacking the majority of these OTUs. The missing OTUs largely belong to the families Lachnospiraceae and Lactobacillaceae. The family Lactobacillaceae OTUs were present to a slightly higher degree during the pre-hibernation, 24 hours post arousal, and 7 day post arousal samples than the Lachnospiraceae OTUs. Additionally, the Lactobacillaceae OTUs diminished to a much lower abundance during hibernation and 2 hours post

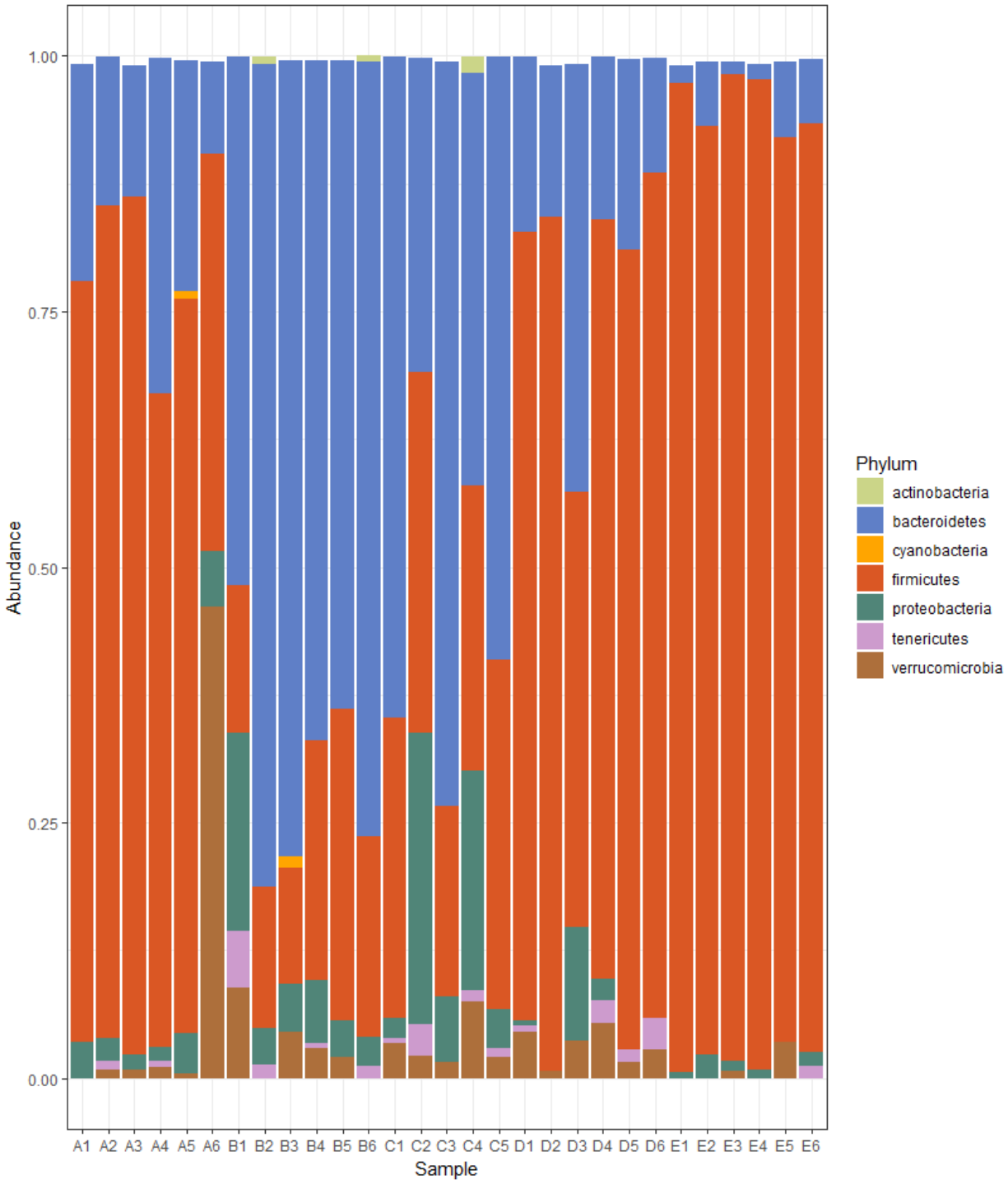

FIG. 7. Relative abundance of bacterial community phyla in cecal contents from thirteen lined ground squirrels prior to hibernation (A1-A6), during inter-bout arousal (B1-B6), and after 2 hours (C1-C5), 24 hours (D1-D6), and 7 days (E1-E6) post arousal. OTUs making up less than 0.5% of the total sequences in a given sample are not shown.

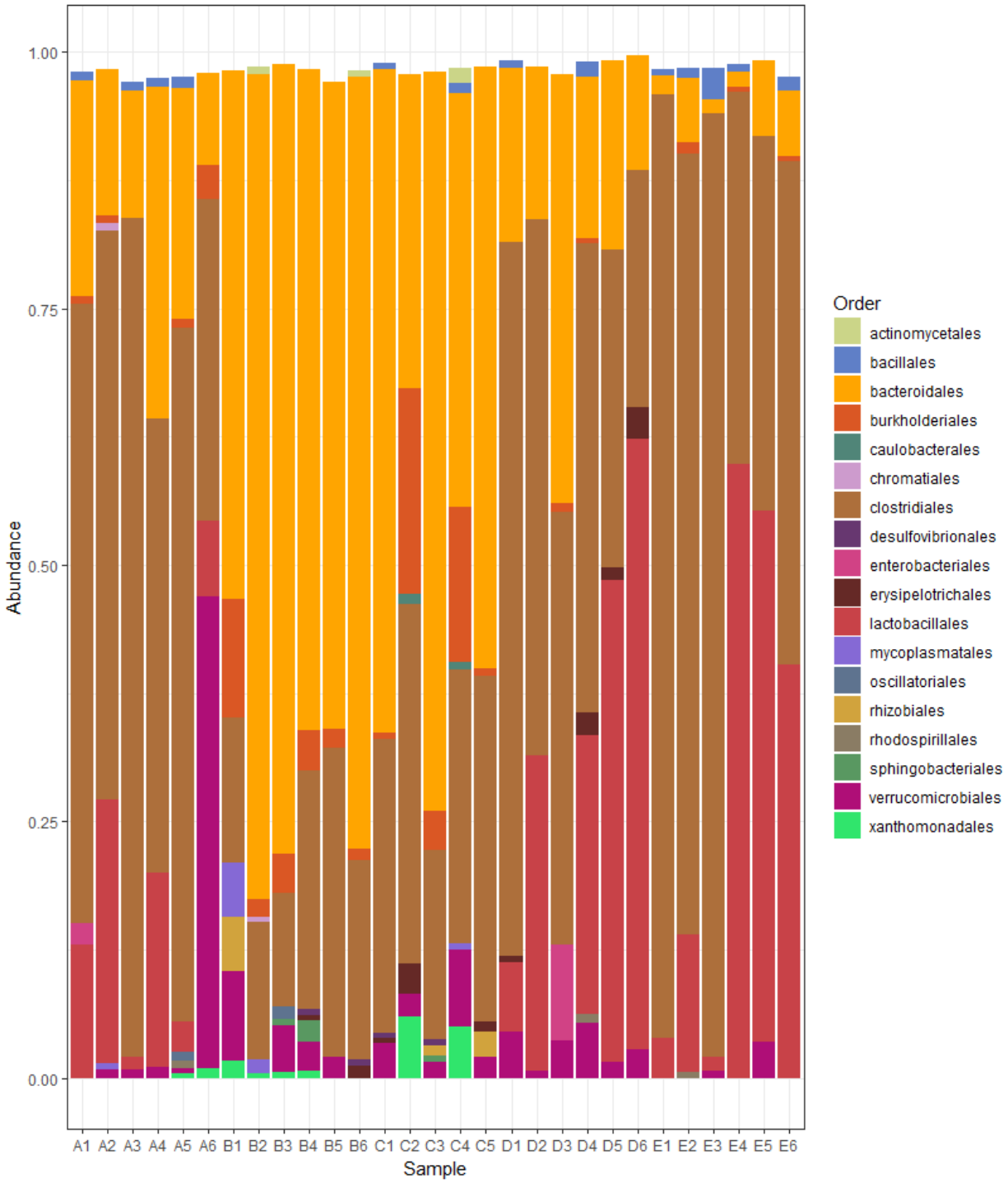

FIG. 8. Relative abundance of bacterial community orders in cecal contents from thirteen lined ground squirrels prior to hibernation (A1-A6), during inter-bout arousal (B1-B6), and after 2 hours (C1-C5), 24 hours (D1-D6), and 7 days (E1-E6) post arousal. OTUs making up less than 0.5% of the total sequences in a given sample are not shown.

arousal than the Lachnospiraceae OTUs. The Firmicutes heatmap suggests that the previous observation of a decrease in Firmicutes abundance during inter-bout arousal and 2 hours post arousal was the result of a decrease in the families Lachnospiraceae and Lactobacillaceae.

In contrast, OTUs present from the phylum Bacteroidetes were not observed to group relative to hibernation stages (Fig. 10). Although abundance of Bacteroidetes was observed to return to similar levels as pre-hibernation at the 24 hour and 7 day post arousal timepoints, the community makeup was different at these stages. The increase seen in Bacteroidetes appeared to be predominantly due to an increase in the family Rikenellaceae (Fig. 11).

Principal component analysis of the cecal communities showed samples from pre-hibernation and 7 days post arousal grouped together, and the samples from inter-bout arousal and 2 hours post arousal grouped together, with the exception of one inter-bout arousal sample (Fig. 12). The samples from 24 hours post arousal did not closely align together or with either of the other groups, indicating a higher level of variability during that stage. Taken together, these groups suggest a cyclical progression where the community begins and ends at a similar composition with a major shift during hibernation that begins to change between 2 and 24 hours post arousal, and finally has returned to starting conditions within 7 days of arousal.

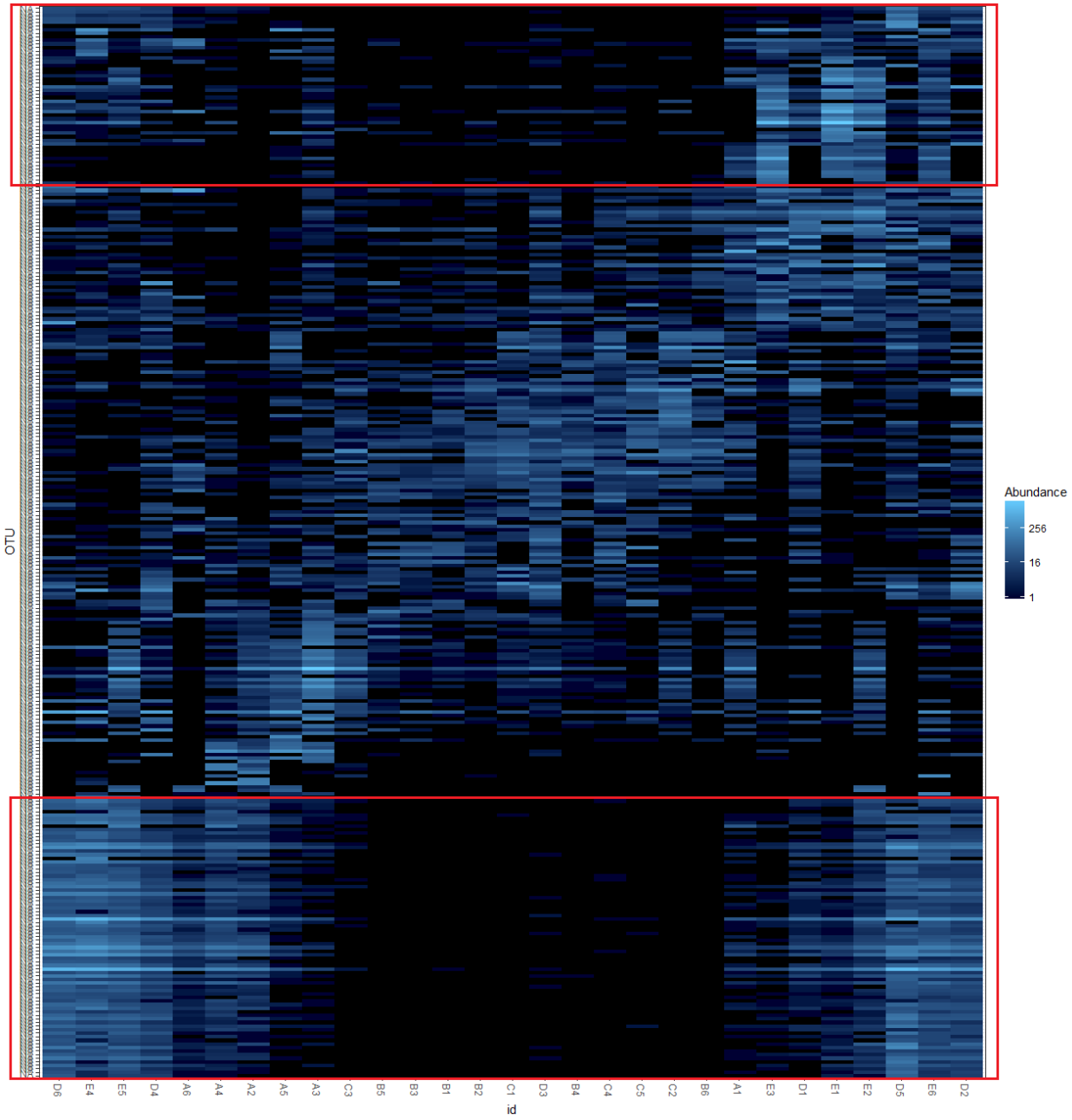

FIG. 9. Heatmap of the top 300 OTUs in the phylum Firmicutes in cecal contents from thirteen lined ground squirrels prior to hibernation (A1-A6), during inter-bout arousal (B1-B6), and after 2 hours (C1-C5), 24 hours (D1-D6), and 7 days (E1-E6) post arousal. OTUs in the upper box primarily belong to the family Lachnospiraceae and OTUs in the lower box primarily belong to the family Lactobacillaceae.

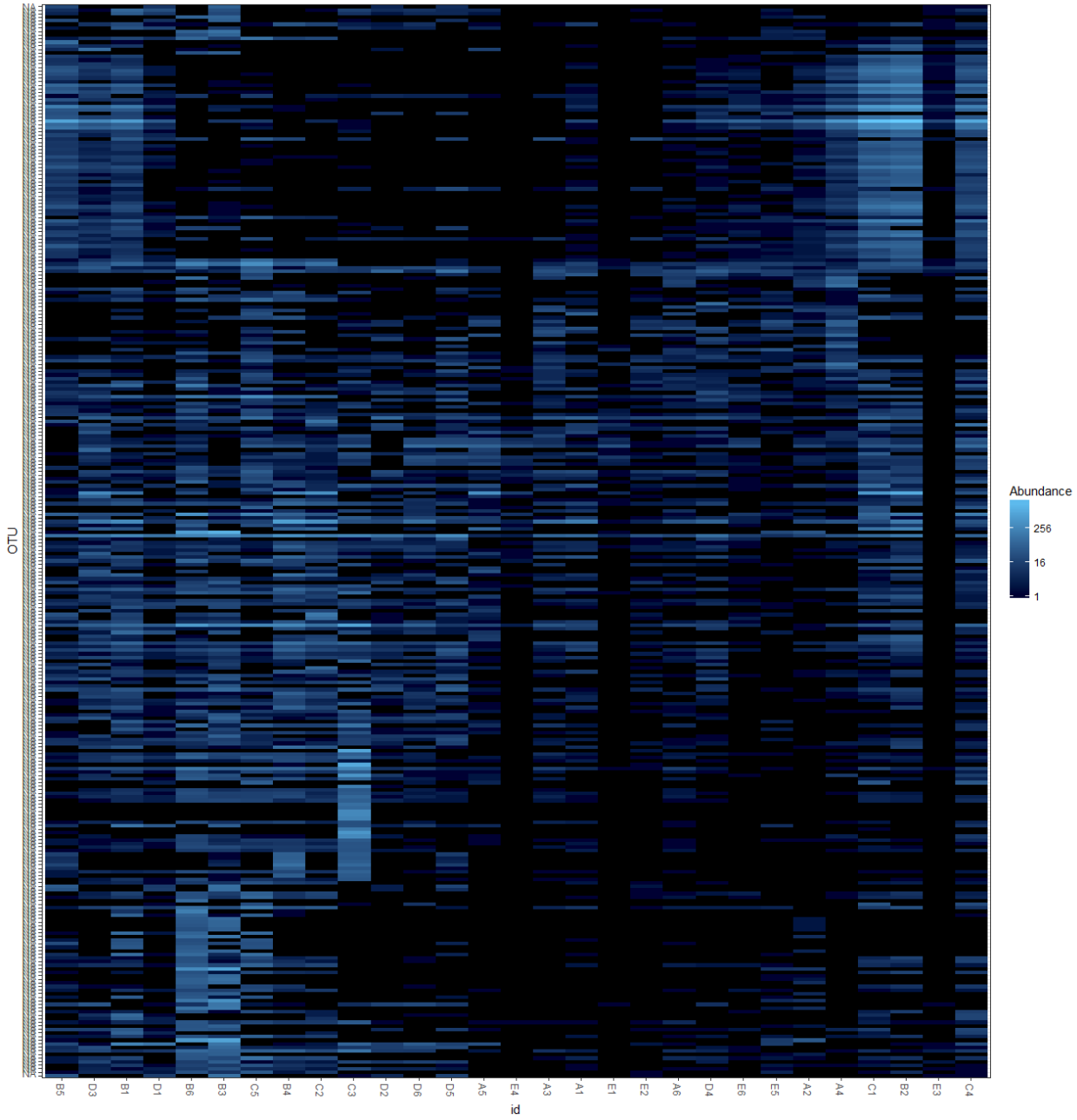

FIG. 10. Heatmap of the top 300 OTUs in the phylum Bacteroidetes in cecal contents from thirteen lined ground squirrels prior to hibernation (A1-A6), during inter-bout arousal (B1-B6), and after 2 hours (C1-C5), 24 hours (D1-D6), and 7 days (E1-E6) post arousal.

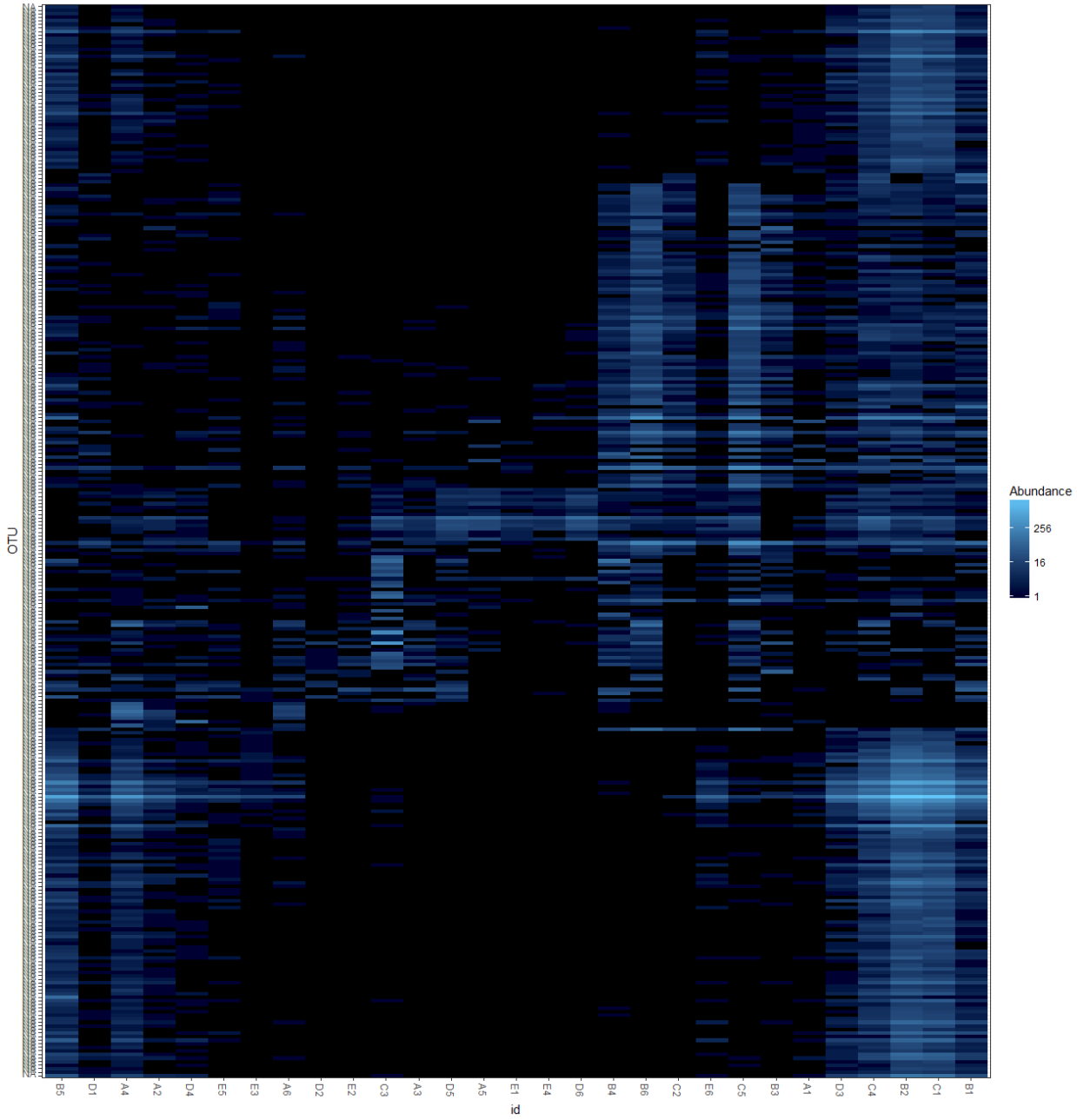

FIG. 11. Heatmap of the top 300 OTUs in the family Rikenellaceae in cecal contents from thirteen lined ground squirrels prior to hibernation (A1-A6), during inter-bout arousal (B1-B6), and after 2 hours (C1-C5), 24 hours (D1-D6), and 7 days (E1-E6) post arousal.

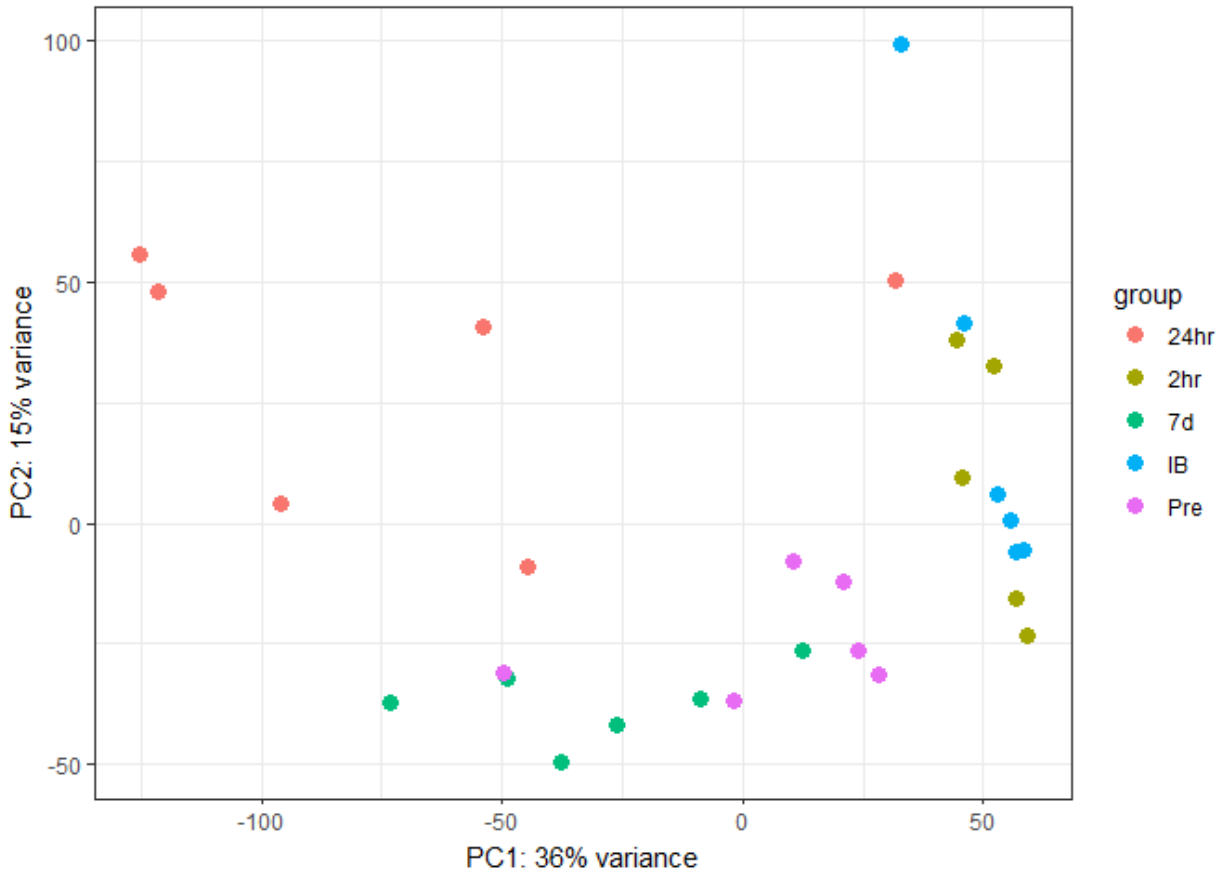

FIG. 12. Principal component analysis comparing Bacterial community composition of the cecal contents from thirteen lined ground squirrels prior to hibernation (Pre), during inter-bout arousal (IB), and after 2 hours (2h), 24 hours (24h), and 7 days (7d) post arousal.

DISCUSSION

The object of this research was to determine how the extreme dietary shift caused by hibernation affected the microbial normal flora cecal community of thirteen-lined ground squirrels with respect to both community composition and abundance, and also included an investigation into the kinetics of these changes.

The effect of hibernation on cecal microbial communities has been studied to an extent in a variety of mammals, including Syrian hamsters (18), arctic ground squirrels (22), and thirteen-lined ground squirrels (23). Additionally, fasting studies investigating cecal bacterial communities have been performed on animals such as Burmese pythons (27), laboratory mice (28), and Syrian hamsters (18). These studies, in particular those involving the species of hibernating squirrels, have identified a common cycle with respect to relative abundance where prior to hibernation animals' cecal communities were predominantly comprised of Firmicutes but a shift occurred during hibernation towards a community dominated by Bacteroidetes. After the animals returned to an active lifestyle, the observed shift was reversed back to a community structure similar to the pre-hibernation samples.

Previous studies, however, have not included direct analysis of microbial abundance while investigating the changes caused by hibernation on cecal communities. In the data from this study, similar to the community structure results, bacterial cell numbers went through a cycle during hibernation. Bacterial cell counts began at a very high value, averaging greater than 4000 cells per mg of cecal material. During the

hibernation stage, and immediately following, this average decreased by greater than 75%. The abundance returned to the same levels as pre-hibernation after transitioning through an intermediate abundance level during the 24 hour post-hibernation time. The significant decrease in microbial abundance is likely the driving force behind shifts in microbial community structure.

The data in this study also identified a similar cycle as previous studies with respect to relative abundance of microbial species. The microbial community composition transitioned from a starting state pre-hibernation, underwent a change during hibernation, and then went through a reverse transition back towards pre-hibernation composition in the hours immediately following arousal from hibernation. Previous studies have not included multiple post-hibernation timepoints, therefore this study provided some additional insight into the kinetics of the changes after hibernation has ended. The reverse transition had not begun by 2 hours post arousal, but was underway at 24 hours post arousal. By one week post arousal the community had shifted back to pre-hibernation state. The identified cycle manifested with a community structure dominated by Firmicutes during the pre-hibernation stage which then shifted to a Bacteroidetes dominated community during and immediately following hibernation, and transitioned back to a Firmicutes dominated structure within one week of arousal. Additionally, although lower in overall abundance, phylum Verrucomicrobia was more predominant in hibernation stages than in the aroused stages.

A noteworthy result from the hibernation stage of the cycle was that *Akkermansia muciniphila* was identified to be the sole OTU from the phylum Verrucomicrobia. *A. muciniphila* was also identified by Carey at. al. as the only Verrucomicrobia member

present in any significant abundance during hibernation stages. *A. muciniphila* is one of few microbes that can degrade mucins (29,30,31) and can utilize mucins as a sole carbon and nitrogen source (29,32). During hibernation no external nutrients would be present in the gut. Mucins, which are glycosylated proteins produced by the epithelial cells of animals, would likely represent the majority of substrates available to microbial normal flora (31,33,34). Organisms that cannot utilize mucins or byproducts from the consumption of mucins would therefore be expected to be absent or greatly decreased during hibernation periods. The order Lactobacillales is an example of a group that grows predominantly on sugars that would no longer be present during hibernation, and thus are conspicuously absent from hibernation stage samples. The appearance of the mucin degrader *A. muciniphila* and shift towards Firmicutes that was observed in hibernation is a pattern that has also been observed in fasting studies of non-hibernating animals. These results have been seen in the Burmese python and laboratory mice, neither of which hibernate, as well as during a fasting active condition in Syrian hamsters, which do hibernate (18). The similar shift in microbial community composition as the result of hibernation or fasting could mean that hibernation might serve as a suitable model to monitor the effect of fasting on intestinal microflora.

Previous research by Carey et. al. (23) observed this common cyclical pattern of community structure during the hibernation process, also using thirteen-lined ground squirrels. The same pattern was identified where Firmicutes were dominant during the active stages while Bacteroidetes and Verrucomicrobia were more dominant during the hibernation stages, but additional timepoints in this study helped elucidate the speed with which the reverse transition occurred.

Some possible explanations for the behavior of this cycle include:

1. Firmicutes decreased in abundance while Bacteroidetes remained the same resulting in a shift towards Bacteroidetes during hibernation stages.
2. Bacteroidetes increased dramatically in abundance while Firmicutes remained the same, resulting in a shift towards Bacteroidetes during hibernation stages.
3. Firmicutes decreased in abundance while Bacteroidetes increased in abundance, resulting in a major shift towards Bacteroidetes during hibernation stages.

When considering the abundance cycle in addition to community structure cycle, the drastic drop in bacterial load would likely make the second scenario incorrect. The fact that there is a fivefold decrease in population from pre-hibernation to during hibernation, as well as the virtual disappearance of the order Lactobacillales, points more towards the first scenario. However, even if the Bacteroidetes population size did not change, it appeared that the community composition did change. It may be possible, though, that the differences seen were due to the fact that the animals had to be sacrificed in order to generate the samples, so analysis had to be done on communities that developed in parallel to each other rather than continuous analysis of a single community. The possibility also remains that the Bacteroidetes population changes in composition, but remains similar in abundance. Again, the nature of comparing parallel communities makes this difficult to determine.

Transition of the microbial community from hibernation back to active stage did not appear to have started by 2 hours post arousal but was well under way by 24 hours. At 7 days the community structure had returned to pre-hibernation state. Given that the transition had already begun by 24 hours it would seem more likely that the return to pre-

hibernation state occurred closer to the 24 hour timepoint than to the 7 day timepoint, however the point at which this reversion was completed would need additional testing to be determined. This study also did not investigate the kinetics of the transition into hibernation. It is possible that a very similar progression occurs immediately following the transition into torpor as we observed after torpor ended.

During hibernation, thirteen-lined ground squirrels go through periods of torpor with instances of inter-bout arousal occurring intermittently. The samples taken during hibernation in this study were during an inter-bout arousal, so the results may not reflect the entirety of hibernation. It is likely that overall abundance of microflora would be even further diminished during the torpor stages, since body temperature of the host squirrel would be as low as 4°C. At this temperature it is likely that metabolic activity of microflora will be at a very low level resulting in minimal activity or growth. It may be possible that a cycle occurred within the hibernation stage itself where community composition and abundance cycled with the stages of torpor and inter-bout arousal. Previous work by Carey et. al. has indicated that species diversity decreases from early hibernation to late hibernation, suggesting that rather than a single shift occurring at the start of hibernation and being maintained, a steady decline may occur throughout the hibernation process.

One cecal sample, from the 2 hour post hibernation stage, had a high level of Enterobacteriales. These OTUs resolved to be entirely *Shigella* species. *Shigella*, a known human pathogen, has not been shown to be a pathogen of thirteen lined ground squirrels. It is unclear, then, where the population of *Shigella* would have come from, and what role, if any, it may play in the gut community. The presence of *Shigella* may simply

come down to sample variance which could be revealed with larger sample sizes.

Hibernation causes a significant shift in the behavior of the thirteen-lined ground squirrel's immune system, specifically greater mucosal levels of certain types of lymphocytes, and increased mucosal IgA levels (35). Interplay between microbial flora and immune system development has been observed, specifically incomplete development of the immune system in sterile conditions (6,7,13). Since immune system development is stunted in the absence of microbes, the change in immune system behavior in the intestines during hibernation may be driven by the radical shift in microflora community. These changes may be driven by the host interaction with the abundant species during hibernation, but since it is known that an absence of microbes has significant impact on the immune system it may be possible that the reduced or altered microbial population during hibernation has an effect as well.

This work expanded upon previous reports detailing the alteration of gut microbial flora of thirteen-lined ground squirrels during hibernation involving culture based techniques (17) as well as deep sequencing of 16S rRNA genes (23). In addition to using 16S rRNA gene-based phylogenetic analysis, the data here included abundance numbers which had not previously been investigated. This information added another layer of detail to the picture of how microbial populations change in hibernation cycles. The abundance data puts forth the question of how does the community restructuring manifest during hibernation: is it a complete restructuring of the entire microflora community, or are certain populations selectively reduced while others remain mostly static? Our belief is that, although relative abundance of Bacteroidetes increased during hibernation conditions, the overall abundance of Bacteroidetes remains similar to active

stage conditions, and the significant change in overall abundance of microflora is driven by the significant reduction in the Firmicutes population. Additionally, our data provided some insight into the speed with which the cecal microflora community of thirteen-lined ground squirrels might return to pre-hibernation status. Significant changes were observed as soon as 24 hours post arousal, with complete return to pre-hibernation state by one week post arousal. Additional sampling of points between one day and one week post arousal may provide a more precise prediction of when the community changes have completed.

Several studies investigating the effect of hibernation on microbial communities have identified a cycle that occurs within the community structure. The cycle was characterized again in this study, and further explored to include investigation of additional timepoints within the cycle as well as an investigation of microbial abundance during this cycle. In this study, the diet of the squirrel hosts was not strictly controlled, which could have played a role in the variability of abundance results. The quantity of material consumed immediately prior to any dissection may play a major role in the microbial abundance. Additionally a study investigating a variety of diet types could elucidate if portions of the microbial population are specifically important to the host or if they are simply a product of one specific diet.

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