Characterization of captive and wild 13-lined ground squirrel cecal microbiotas using Illumina-based sequencing

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Abstract

Background

Hibernating animals experience extreme changes in diet that make them useful systems for understanding host-microbial symbioses. However, most of our current knowledge about the hibernator gut microbiota is derived from studies using captive animals. Given that there are substantial differences between captive and wild environments, conclusions drawn from studies with captive hibernators may not reflect the gut microbiota's role in the physiology of wild animals. To address this, we used Illumina-based sequencing of the 16S rRNA gene to compare the bacterial cecal microbiotas of captive and wild 13-lined ground squirrels (TLGS) in the summer. As the first study to use Illumina-based technology to sequence the microbiotas of an obligate rodent hibernator, we also reported changes in captive TLGS microbiotas across the year (summer, winter, and spring).

Results

Wild TLGS microbiotas had greater richness and phylogenetic diversity with less variation in beta diversity when compared to captive microbiotas. Taxa identified as core operational taxonomic units (OTUs) and found to significantly contribute to differences in beta diversity were primarily in the families *Lachnospiraceae* and *Ruminococcaceae*. Captive TLGS microbiotas shared phyla and core OTUs across the year, but active season (summer and spring) microbiotas had different alpha and beta diversities than winter season microbiotas.

Conclusions

This is the first study to compare the microbiotas of captive and wild rodent hibernators. Our findings suggest that data from captive and wild ground squirrels should be interpreted separately due to their distinct microbiotas. Additionally, as the first study to investigate the microbiotas of obligate rodent hibernators using Illumina-based 16S rRNA sequencing, we reported seasonal changes in captive TLGS microbiotas that are consistent with previous work. Taken together, this study provides foundational information for improving the reproducibility and experimental design of future hibernation microbiota studies.

Introduction

Host-microbe symbioses are dynamic, especially in animals that experience extreme shifts in diet as these changes dramatically alter substrate availability for their gut microbiota. An example includes hibernating mammals. Hibernation is an ecophysiological strategy to survive times of reduced resource availability and high-energy demand by altering both behavior and physiology. In small mammalian hibernators such as the 13-lined ground squirrel (TLGS; *Ictidomys tridecemlineatus*), the circannual

hibernation cycle involves periods of summer hyperphagia when the host acquires adequate fat stores for energy usage during winter hibernation [1]. During summer, the gut microbiota has access to both dietary and host-derived substrates as rich sources of energy. In winter, the host enters hibernation, fasts for several months, and relies entirely upon fat stores for energy. The winter hibernation season is characterized by cycling between periods of depressed and normal metabolism (torpor and interbout arousal, respectively). Torpor occurs when metabolism slows to < 4% of normal rates, causing body temperature (T_b) to plummet to just above ambient temperature (< 10°C) [2, 3]. Torpor is interrupted by brief interbout arousals (IBAs) that can last 12–24 hours [2–4]. During IBAs, metabolism increases to normal rates and T_b warms to ~ 36°C [2–4]. Due to fasting during hibernation, the gut microbiota is forced to rely solely on host-derived substrates (e.g., mucins) as its source of energy. The hibernation season ends with the emergence aboveground in spring and the resumption of normal metabolic activity and feeding patterns. This natural cycle of extreme changes in diet and physiology makes mammalian hibernators like the TLGS useful systems for studying host-microbe symbioses.

The majority of our current knowledge of the hibernator gut microbiota comes primarily from studies of captive animals. Captive environments differ from natural, wild environments due to necessary changes in diet, habitat, rearing conditions, and exposure to environmental microbes [5]; as a result, the microbiotas of captive animals likely differ substantially from those of wild animals. While the use of captive animals allows for controlled experiments that have led to significant advances in our understanding of their biology, their use for gut microbiota studies reduces confidence in the conclusions that can be drawn about the microbiota's role in the physiology of wild animals. Moreover, these limitations may restrict the ability to translate discoveries using captive host-microbial relationships to other systems, such as the use of captive mice as a model for human health [5–8]. Differences in diet are of particular concern as diet is a known major driver of microbiota composition and metabolism [9–17] and it is difficult to recapitulate a hibernator's wild diet in a captive setting. Additionally, the development of an animal's microbiota early in life can have long-lasting effects on microbiota composition and host physiology [13, 18, 19], and many hibernation studies use animals born in the lab rather than in the wild. Although there is a growing number of studies comparing the microbiotas of captive and wild animals [5–8, 20–30], very few have been conducted with hibernating species. One study that examined three species of hibernating bats found that the number of operational taxonomic units (OTUs) is higher in captive than in wild bats [28]. Studies in other animals have reported that differences between captive and wild microbiotas are species-specific [5]. Therefore, it is important to understand how captivity alters the microbiota of hibernating animals like the TLGS.

Here, we used Illumina 16S rRNA gene sequencing to compare the bacterial cecal microbiotas of captive and wild TLGS in the summer. We hypothesize that wild TLGS microbiotas are more diverse than captive TLGS microbiotas, likely because the wild diet is more diverse than the captive diet. As this study is, to our knowledge, the first analysis of Illumina-based sequencing from an obligate rodent hibernator, we also reported changes in captive TLGS microbiotas across the year (summer, winter, and spring).

Methods

Animals

All procedures were approved by the University of Wisconsin-Madison Institutional Animal Care and Use Committee under protocols V001229 and V005481. Two groups of healthy TLGS were used in our study: a captive and a wild group (Fig. 1). The captive group consisted of 26 animals (17 females and 9 males) that were born in captivity to seven pregnant, wild-caught females in Madison, WI. The pregnant females were housed individually at 22°C with a 12:12hr light-dark cycle, provided water and rat chow (Harlan Teklad no. 7001, Indianapolis, IN, USA) ad libitum, and supplemented with apples, strawberries, and sunflower seeds weekly. Pups were born in May 2016 and remained with their mothers for five weeks before separation into new cages that housed two pups per cage. After two weeks, pups were transferred into individual cages. Water and chow were provided ad libitum for two weeks. Then chow was restricted to 12 g/day to prevent excessive weight gain and sunflower seeds (~ 1 g) were provided weekly. The wild squirrel group consisted of four adult TLGS (two males and two females) captured in July 2017 in Madison, WI.

Sampling Regimen

The 26 captive TLGS were randomly assigned to one of four groups (Fig. 1): Summer ($n = 8$), Torpor ($n = 6$), IBA ($n = 8$), or Spring ($n = 4$). Captive Summer squirrels were sampled in August 2016. The remaining captive animals were transferred to a 4°C room in September/October 2016 for hibernation. The room was held in constant darkness except for a daily, brief period of dim light (~ 5 min) to check activity states using the sawdust method [31]. Once squirrels began using torpor, food and water were removed. Winter squirrels were sampled in January – February (after ~ 3.5–4.5 months in the cold room). Torpor squirrels were sampled during torpor ($T_b < 10^\circ\text{C}$). For IBA squirrels, torpid animals were brought to a lit 22°C room for three hours to induce an arousal and sampled when $T_b > 34^\circ\text{C}$. Spring squirrels were removed from the cold room in January 2017 and transferred to a warm room with food and water ad libitum for two weeks before sampling in February 2017. Samples were collected from wild summer squirrels on the same day they were captured in July 2017. Metadata for all squirrels are listed in Table S1.

Sample collection

Four anesthesia/euthanasia methods were used due to equipment availability and sample collection requirements for a separate project. Captive Summer and Spring groups were euthanized via exposure to isoflurane for 5–15 min followed by decapitation. Torpor squirrels were euthanized by decapitation or cervical dislocation. IBA squirrels were anesthetized via exposure to isoflurane or CO₂ for 5–15 min followed by decapitation (Table S1). Wild summer squirrels were euthanized with pentobarbital followed by decapitation. To determine whether anesthesia method had a significant impact on the gut microbiota, IBA squirrels that received isoflurane or CO₂ were compared as this was the only experimental group

where multiple anesthesia methods were used (Table S2). We did not find evidence that the microbiotas of isoflurane- or CO₂-administered animals were different. The methods and results for this analysis are shown in Table S2.

T_b was measured immediately by inserting a clean thermal probe into the body cavity. Cecal contents were collected by removing intact ceca and gently scraping the content into a sterile tube. To remove any remaining content, cecal tissue was rinsed with sterile phosphate-buffered saline (PBS) and any remaining liquid was gently scraped off. Cecal mucosae were collected using a razor blade to gently scrape off the mucosa. To assess the captive diet microbiota, three chow samples were collected. A representative wild diet was not collected as TLGS are omnivorous and consume a diverse diet that includes various plants, insects, and small birds [32]. All samples were placed on dry ice and stored at -80°C. We collected a total of 62 samples: 30 cecal content samples (26 captive, 4 wild), 29 cecal mucosa samples (25 captive, 4 wild), and three chow samples.

DNA Extraction

Total genomic DNA was extracted from each sample using a phenol:chloroform extraction protocol [33] with the following modification: all aqueous phase washes used 25:24:1 phenol:chloroform:isoamyl alcohol instead of phenol:chloroform for a total of four washes. Four controls were processed with the cecal samples. Two sample collection controls were aliquots from the sterile PBS used to rinse cecal tissue. These were uncovered and exposed to air for the same amount of time needed to collect the cecal samples. Two extraction method controls contained sterile water. DNA was quantified with the Qubit Fluorometer (Invitrogen, San Diego, CA, USA) and stored at -80°C.

DNA amplification and sequencing

We used universal bacterial primers flanking the V4 region of the 16S rRNA gene (forward: GTGCCAGCMGCCGCGGTAA, reverse: GGACTACHVGGGTWTCTAAT) [34]. The primers also contained adapters (forward: AATGATACGGCGACCACCGAGATCTACAC, reverse: CAAGCAGAAGACGGCATACGAGAT) compatible with Illumina sequencing technology (Illumina, San Diego, CA, USA) and unique barcodes for multiplexing (forward: eight unique eight bp barcodes, reverse: 12 unique eight bp barcodes). Each reaction contained 50 ng DNA, 0.4 µM forward primer, 0.4 µM reverse primer, 12.5 µL 2X HotStart ReadyMix (KAPA Biosystems, Wilmington, MA, USA), and water to a final volume of 25 µL. Polymerase chain reaction (PCR) was performed using a Bio-Rad S1000 thermocycler (Bio-Rad Laboratories, Hercules, CA, USA). Cycling conditions began with initial denaturation at 95°C for 3 min, followed by 25 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s, and a final extension at 72°C for 5 min. Four controls consisting of sterile water were processed alongside sample DNA to ensure there was no contamination during PCR. PCR products were purified using gel extraction from a 1.0% low-melt agarose gel (National Diagnostics, Atlanta, GA, USA) with a ZR-96 Zymoclean DNA Recovery Kit (Zymo Research, Irvine, CA, USA) and DNA was quantified using a Qubit Fluorometer. 60 samples were successfully amplified, and two samples failed to amplify (both chow samples). Samples were equimolarly pooled with 10% PhiX control DNA and sequenced on an Illumina MiSeq using a MiSeq 2 x 250 v2 kit.

Microbiota sequence clean-up

Sequences were demultiplexed on the Illumina MiSeq and processed using mothur v1.44.3 [35] and SILVA release 132 [36–39]. Fastq files were submitted to NCBI's Short Read Archive and are publicly available under accession number PRJNA742778. Mothur logfiles and output files are found at https://github.com/ednachiang/CaptiveWild/mothur_outputs.

We followed the mothur SOP accessed on March 19, 2021 with the following modifications: we used *screen.seqs* maxlength = 300 and *chimera.uchime*. Sequences were grouped into 97% operational taxonomic units (OTUs). Samples were normalized to 11,013 sequences. This cutoff was selected based on the sample with the lowest number of sequences that resulted in all normalized samples having Good's coverage $\geq 95\%$, which indicated sufficient sequencing [40]. Three samples were removed due to insufficient sequencing (Good's coverage $< 95\%$): one chow, one Torpor mucosa, and one summer Wild content sample. All eight control samples were also discarded due to low number of sequences (mean = 32; range = 3–117). To create a phylogenetic tree, a representative sequence from each OTU was first selected using *get.oturep* and the most abundant sequence. Representative sequences were renamed to match their assigned OTU and a phylip-formatted distance matrix was calculated. This distance matrix was used to calculate a phylogenetic tree using *clearcut* and the tree was classified using *classify.tree*.

Preparing data for statistical analysis in R

Outputs from mothur were imported into R version 3.4.3 [41] using the phyloseq [42] and phytools [43] packages. All visualizations were created using the ggplot2 [44], grid [41], and gridExtra [45] packages. The following packages were also used for statistical analyses: ape [46], dplyr [47], dunn.test [48], equivalence [49], picante [50], stats [41], tidyr [51], and vegan [52].

We used different subsets of samples to perform three analyses. To compare cecal content and mucosa microbiotas, only squirrels with both sample types were included due to the use of paired tests, and samples within each experimental group were analyzed separately. To compare Captive and Wild microbiotas, only summer Captive and summer Wild squirrels were considered, and content and mucosa samples were analyzed separately. Lastly, to compare captive microbiotas across the year (summer, winter, and spring), we analyzed captive groups (Summer, Torpor, IBA, and Spring) separately for content and mucosa samples. In all three comparisons, OTUs found in less than three samples were removed. Four outlier samples were also removed: content and mucosa samples from one IBA and one Spring individual. All code to replicate statistical analyses and generate figures is available at <https://github.com/ednachiang/CaptiveWild>.

Alpha diversity analysis

We evaluated alpha diversity (within-sample diversity) using the number of unique OTUs, Faith's phylogenetic diversity, phylogenetic evenness, and Shannon's weighted diversity index [53]. The number of unique OTUs was calculated using the phyloseq package [42]. Faith's phylogenetic diversity was computed by taking the sum of all branches in a tree using the picante package (*pd*) [50]. Because

phylogenetic diversity is positively correlated with richness, we included a metric unbiased by richness: mean pairwise distance (MPD), which measures phylogenetic evenness [54]. MPD represents the average phylogenetic distance between all species pairs. Positive values indicate more phylogenetic evenness while negative values indicate less phylogenetic evenness. MPD was calculated by comparing phylogenetic diversity to a null model over 999 iterations (*cophenetic*, stats package [41]; *ses.mpd*, picante package [50]). Lastly, Shannon's weighted diversity index was calculated in *mothur* [35]. We tested the normality of each alpha diversity metric using quantile-quantile plots and the Shapiro test (*qqnorm*, *qqline*, *shapiro.test*, stats package [41]) before selecting a statistical test.

To compare content and mucosa microbiotas, paired t-tests were used as all metrics had normal distributions. Similarly, t-tests were used to compare Captive and Wild microbiotas. To compare captive microbiotas across seasons, content samples were analyzed using analysis of variance (ANOVA) (*aov*, stats package [41]) followed by pairwise Tukey's Honest Significant Difference tests (*TukeyHSD*, stats package [41]). Mucosa MPD and Shannon's diversity index were also analyzed this way. Mucosa number of unique OTUs and phylogenetic diversity were not normally distributed and were therefore analyzed using Kruskal-Wallis (*kruskal.test*, stats package [41]) followed by Dunn's Test (*dunn.test*, dunn.test package [48]). All p-values were adjusted for false discovery rate using the Benjamini-Hochberg procedure (*p.adjust*, stats package [41]). Results were considered significant if $\text{adj } P < 0.05$ except for Dunn's test where $\text{adj } P \leq 0.025$ was significant.

Beta diversity analysis

Beta diversity (between-sample diversity) was examined using weighted UniFrac, unweighted UniFrac, and Bray-Curtis dissimilarity. All three metrics were calculated using the phyloseq package (*distance*) [42] and visualized using principal coordinate analysis (PCoA) ordinations (*ordinate*, phyloseq package [42]). To test whether there were significant differences in variance/dispersion, homogeneity of groups dispersions tests (*betadisper*, vegan package [52]) were used. Next, we tested if beta diversity centroids differed between experimental groups using permutational multivariate analysis of variance [55] (PERMANOVA; *adonis*, vegan package [52]). All p-values were corrected for false discovery rate using the Benjamini-Hochberg procedure.

To identify which OTUs significantly contributed to differences in Bray-Curtis dissimilarity, the similarity percentages test (SIMPER; *simper*, vegan package [52]) was used along with the Kruskal-Wallis test with false discovery rate correction using the Benjamini-Hochberg procedure. Only OTUs that accounted for $\geq 1\%$ of the differences in beta diversity with $\text{adj } P < 0.05$ were considered.

Phylum-level analysis

Phylum-level relative abundances were calculated using the phyloseq [42] and dplyr [47] packages and phyla with total relative abundances $< 1\%$ were removed. Prior to selecting a statistical test, we evaluated normality using quantile-quantile plots and Shapiro tests. For the Captive and Wild comparison, phyla with normal distributions were analyzed using t-tests, whereas those with non-normal distributions were analyzed using Wilcoxon Rank Sum tests (*wilcox.test*, stats package [41]). To compare captive

microbiotas across seasons, phyla with normal distributions were analyzed using ANOVA followed by Tukey's HSD and those with non-normal distributions were analyzed using Kruskal-Wallis followed by Dunn's test. All p-values were corrected for false discovery rate using the Benjamini-Hochberg procedure and results were considered significant if $\text{adj } P < 0.05$, except for Dunn's test where $\text{adj } P \leq 0.025$ was significant (Table S4).

Identify Core OTUs

Core OTUs were defined as OTUs that were present in every sample within a group and were identified using the phyloseq package [42] (Table S8). Core OTUs shared among groups were identified by taking the intersect of the core OTUs in two or more groups.

Results

Sequence coverage

We sequenced a total of 59 samples, which consisted of 30 cecal content and 29 cecal mucosa. Bacterial amplicon sequencing generated a total of 3,252,557 raw sequences with an average of $54,209 \pm 16,411$ sequences per sample (mean $\pm$ SE; range = 534–972,021). Sequence clean-up in mothur resulted in a total of 2,211,103 sequences for an average of $36,852 \pm 13,449$ sequences per sample (range = 291–796,002). After normalization, 57 samples remained (29 content, 28 mucosa) with a range of reads = 10,453–11,159. All samples had Good's coverage $\geq 95\%$, which indicated sufficient sequence coverage [40].

Cecal content and mucosa microbiotas are similar

To determine whether cecal content and mucosa microbiotas differ, we used paired tests to examine alpha and beta diversity within each of the five experimental groups. There were no differences in the number of unique OTUs, Faith's phylogenetic diversity, phylogenetic evenness, or Shannon's diversity (all paired t-test $\text{adj } P \geq 0.889$, Fig. S1). Similarly, we found no differences in the variances of weighted UniFrac, unweighted UniFrac, and Bray-Curtis dissimilarity (all $\text{betadisper adj } P \geq 0.293$; Fig. S2). We then examined the centroids of the three beta diversity metrics by including both squirrel ID and sample type in PERMANOVA tests. Squirrel ID accounted for the paired nature of the data and was significant for all three metrics in the captive summer group (all PERMANOVA $\text{adj } P = 0.002$) and for Bray-Curtis dissimilarity in the IBA group ($\text{adj } P = 0.006$), but not in the remaining groups and metrics. After accounting for squirrel ID, there were no differences in sample type for the three metrics (all $\text{adj } P \geq 0.912$). Because sample type did not significantly impact alpha and beta diversity, we chose to present data from content microbiotas in this present paper; mucosa data are found in the supplementary materials.

Captive and Wild microbiotas have different alpha and beta-diversities

We examined alpha diversity (within-sample diversity) between summer Captive and Wild groups by using four metrics (Fig. 2). In content microbiotas, the number of unique OTUs was higher in Wild compared to Captive (Fig. 2A; T-test adj $P < 0.001$). Analysis of Faith's phylogenetic diversity, which is positively correlated with richness, revealed the same difference as observed for number of unique OTUs (Fig. 2B; adj $P < 0.001$). There were no differences in phylogenetic evenness (Fig. 2C; adj $P = 0.105$) or Shannon's diversity (Fig. 2D; adj $P = 0.940$). The same results were seen in mucosa microbiotas (Fig. S3).

We examined beta diversity by using weighted UniFrac, unweighted UniFrac, and Bray-Curtis dissimilarity (Fig. 3). The variances of all three metrics in content microbiotas were different between Captive and Wild groups (Fig. 3; all betadisperm adj $P = 0.006$). The centroids of all three metrics were also different between Captive and Wild groups (Fig. 3; all PERMANOVA adj $P = 0.014$). Mucosa microbiotas (Fig. S4) had different variances in only unweighted UniFrac (adj $P = 0.012$) whereas their centroids in all three metrics were different (all adj $P = 0.004$).

Few OTUs drive beta diversity differences between Captive and Wild microbiotas

To identify specific taxa that contributed to the differences between Captive and Wild microbiotas, we first examined phyla relative abundances. There were eight phyla with total relative abundances $> 1\%$ across all content samples (Table 1). Dominant phyla included the *Firmicutes* and *Bacteroidetes*, with smaller contributions from the *Verrucomicrobia*, *Proteobacteria*, *Cyanobacteria*, *Tenericutes*, *Actinobacteria*, and *Elusimicrobia*. Two additional phyla were detected in mucosa microbiotas (Table S3): the *Epsilonbacteraeota* and *Kiritimatiellaeota*. Both content and mucosa microbiotas had no differences in phyla relative abundances between Captive and Wild microbiotas (all adj $P > 0.050$); however, the phyla *Proteobacteria* and *Cyanobacteria* displayed a trend in content microbiotas (both adj $P = 0.055$).

Table 1
Phyla relative abundances in summer Captive and Wild content microbiotas. Phyla have total relative abundances > 1% and are displayed from overall highest relative abundance to lowest relative abundance. Relative abundances are displayed as mean $\pm$ standard error.

Relative Abundance (%)			
Phylum	Captive	Wild	Adj P
<i>Firmicutes</i>	67.222 $\pm$ 3.315	57.922 $\pm$ 4.999	0.444
<i>Bacteroidetes</i>	24.133 $\pm$ 2.934	30.048 $\pm$ 5.047	0.484
<i>Verrucomicrobia</i>	3.556 $\pm$ 1.534	2.366 $\pm$ 0.875	1.000
<i>Proteobacteria</i>	2.625 $\pm$ 0.451	1.106 $\pm$ 0.060	0.055
<i>Cyanobacteria</i>	0.455 $\pm$ 0.191	4.701 $\pm$ 0.7062	0.055
<i>Tenericutes</i>	0.675 $\pm$ 0.268	0.992 $\pm$ 0.462	0.484
<i>Actinobacteria</i>	0.292 $\pm$ 0.097	0.368 $\pm$ 0.117	0.720
<i>Elusimicrobia</i>	0.008 $\pm$ 0.004	0.497 $\pm$ 0.250	0.444
Unclassified	0.989 $\pm$ 0.452	1.880 $\pm$ 1.216	0.484

As we saw no differences at the phylum-level, we proceeded at the OTU-level. Analysis of similarity percentages (SIMPER) in content microbiotas identified four OTUs that contributed to differences in Bray-Curtis dissimilarity (Table 2). They explained 7.260% (cumulative SIMPER) of the difference in Bray-Curtis dissimilarity. Three of these OTUs belonged to the family *Lachnospiraceae*, one of which was further classified to the genus *Lachnospiraceae NK4A136 group*, and were not detected in Captive microbiotas (Table 2). The remaining OTU was classified to the genus *Lactobacillus* and had higher relative abundance in Captive than in Wild (Table 2).

Table 2
OTUs that significantly contribute to Bray-Curtis dissimilarity between summer Captive and Wild content microbiotas.

OTU	Most Resolved Taxonomic Classification	SIMPER Percentage	Captive Relative Abundance	Wild Relative Abundance	Adj P
Otu00035	<i>Lactobacillus</i>	3.418%	5.716%	0.047%	0.014
Otu00119	<i>Lachnospiraceae</i>	1.366%	0.000%	2.266%	0.008
Otu00163	<i>Lachnospiraceae NK4A136 group</i>	1.236%	0.000%	2.051%	0.008
Otu00166	<i>Lachnospiraceae</i>	1.240%	0.000%	2.056%	0.008

Mucosa microbiotas had three OTUs that explained 4.716% of the Bray-Curtis dissimilarity (Table S4). Two OTUs were classified to the family *Lachnospiraceae* and one to the genus *Lactobacillus*. Both *Lachnospiraceae* OTUs had higher relative abundances in Wild compared to Captive, whereas the opposite was true for the *Lactobacillus* OTU. Two OTUs (one *Lachnospiraceae* and one *Lactobacillus*) were significant in both content and mucosa microbiotas.

Wild microbiotas have more core OTUs than Captive microbiotas

The Wild content microbiota had 323 core OTUs whereas the Captive content microbiota had 37 (Table S5). The majority of the Wild core OTUs were classified to the families *Ruminococcaceae* (92 OTUs), *Lachnospiraceae* (83 OTUs), *Muribaculaceae* (36 OTUs), and unclassified *Gastranaerophilales* (26 OTUs). The majority of the Captive core OTUs were classified to the families *Ruminococcaceae* (12 OTUs) and *Lachnospiraceae* (7 OTUs). There were 19 shared core OTUs across all content samples (Table S5). All but two of these were classified to the order *Clostridiales* and the most common family was the *Ruminococcaceae* with 12 OTUs. The two non-Clostridia OTUs were respectively classified to the family *Paracaedibacteraceae* and the genus *Lactobacillus*. Mucosa microbiotas had similar numbers and taxonomic classifications of core OTUs (Table S6).

Captive microbiotas across seasons have similar phyla and core OTUs

Across all content samples in the four captive groups, we identified nine phyla with total relative abundances > 1% (Table 3). Only the *Firmicutes* and *Bacteroidetes* differed between groups. The *Firmicutes* had higher relative abundances in Summer compared to Torpor or IBA (adj P = 0.0012 and 0.001, respectively); the opposite was true for the *Bacteroidetes* (Summer and Torpor adj P = 0.006, Summer and IBA adj P = 0.017). Phyla with no differences between groups included the *Verrucomicrobia*, *Proteobacteria*, *Kiritimatiellaeota*, *Cyanobacteria*, *Tenericutes*, *Actinobacteria*, and *Elusimicrobia*. Mucosa microbiotas had the same nine phyla in addition to the phylum *Epsilonbacteraeota* (Table S7). In these samples, only the *Firmicutes* differed, with higher relative abundance in Summer compared to Torpor or IBA (both adj P < 0.001). No other phyla differed between groups.

Table 3

Phyla relative abundances in content microbiotas of captive groups. Phyla have total relative abundances > 1% and are displayed from overall highest relative abundance to lowest relative abundance. Relative abundances are displayed as mean $\pm$ standard error.

Relative Abundance (%)					
Phylum	Summer	Torpor	IBA	Spring	Significant Comparisons
<i>Firmicutes</i>	67.643 $\pm$ 3.240	25.470 $\pm$ 3.823	24.308 $\pm$ 2.734	53.036 $\pm$ 4.454	Summer-Torpor Summer-IBA
<i>Bacteroidetes</i>	23.691 $\pm$ 2.845	44.758 $\pm$ 6.613	41.522 $\pm$ 2.764	39.966 $\pm$ 3.314	Summer-Torpor Summer-IBA
<i>Verrucomicrobia</i>	3.494 $\pm$ 1.522	19.951 $\pm$ 7.257	27.491 $\pm$ 6.634	4.132 $\pm$ 1.673	—
<i>Proteobacteria</i>	2.555 $\pm$ 0.425	2.464 $\pm$ 0.274	2.869 $\pm$ 0.880	1.244 $\pm$ 0.423	—
<i>Kiritimatiellaeota</i>	0.020 $\pm$ 0.020	3.780 $\pm$ 2.424	1.067 $\pm$ 0.761	0.359 $\pm$ 0.293	—
<i>Cyanobacteria</i>	0.450 $\pm$ 0.187	0.895 $\pm$ 0.222	0.454 $\pm$ 0.249	0.176 $\pm$ 0.122	—
<i>Tenericutes</i>	0.710 $\pm$ 0.273	0.132 $\pm$ 0.057	0.142 $\pm$ 0.0311	0.743 $\pm$ 0.362	—
<i>Actinobacteria</i>	0.305 $\pm$ 0.443	0.160 $\pm$ 0.047	0.138 $\pm$ 0.031	0.091 $\pm$ 0.041	—
<i>Elusimicrobia</i>	0.008 $\pm$ 0.005	0.075 $\pm$ 0.048	0.384 $\pm$ 0.213	0.003 $\pm$ 0.003	—
<i>Unclassified</i>	1.100 $\pm$ 0.461	2.311 $\pm$ 2.113	1.622 $\pm$ 1.248	0.245 $\pm$ 0.118	—

In the captive content microbiotas, we identified 37 core OTUs in Summer, 57 in Torpor, 54 in IBA, and 132 in Spring (Table 4). Core OTUs in each group were dominated by the families *Lachnospiraceae* and *Ruminococcaceae* (Table S8). Torpor and Spring also had > 10 core OTUs classified to the family *Muribaculaceae*. There were 15 core OTUs found in every content sample. The mucosa microbiotas had similar results (Table S9), and 10 core OTUs found in all mucosa samples were also found in all content samples.

Table 4
The number of core OTUs in content microbiotas of each captive group and shared between groups.

	Summer	Torpor	IBA	Spring
Summer	37	21	25	26
Torpor		57	32	40
IBA			54	33
Spring				132

Captive microbiotas in active and winter groups have different alpha and beta-diversities

Summer content microbiotas had more unique OTUs (Fig. 4A) compared to Torpor or IBA (Tukey's Honest Significant Test adj $P = 0.010$ and 0.026 , respectively), but did not differ from Spring (adj $P = 0.420$). Torpor was not different from IBA or Spring (adj $P = 0.952$ and 0.636 , respectively), and IBA was not different from Spring (adj $P = 0.852$). Although phylogenetic diversity (Fig. 4B) is positively correlated with richness, it only displayed a trend of differences between groups (adj $P = 0.069$). Phylogenetic evenness (Fig. 4C) was lower in Summer compared to Torpor or IBA (both adj $P < 0.001$), and lower in Spring compared to Torpor (adj $P = 0.042$). There were no differences between Summer and Spring (adj $P = 0.088$), Torpor and IBA (adj $P = 0.958$), or IBA and Spring (adj $P = 0.083$). Shannon's diversity index did not differ between groups (Fig. 4D, adj $P = 0.793$). Mucosa microbiota results (Fig. S5) were the same as for the content microbiota with respect to the number of unique OTUs and Shannon's diversity index; however, phylogenetic diversity and phylogenetic evenness differed. Phylogenetic diversity was higher in Summer than IBA (adj $P = 0.018$), and phylogenetic evenness was lower in Summer than Torpor (adj $P = 0.022$).

Beta diversity variance across all captive content microbiotas (Fig. 5) did not differ in weighted and unweighted UniFrac and in Bray-Curtis dissimilarity (all betadisper $P \geq 0.174$). However, the centroids of active groups (Summer and Spring) were different from those of winter groups (Torpor and IBA; PERMANOVA adj P -values for weighted UniFrac < 0.017 , unweighted UniFrac < 0.038 , and Bray-Curtis dissimilarity < 0.029). There were no differences within active and winter groups (all adj P -values for weighted UniFrac > 0.064 , unweighted UniFrac > 0.231 , and Bray-Curtis dissimilarity > 0.131). Mucosa microbiota beta diversity results (Fig. S6) were similar to those of content microbiotas, except that the unweighted UniFrac centroids of Torpor and Spring were not different (adj $P = 0.105$). Lastly, we attempted to identify OTUs that drove these differences in beta diversity by using SIMPER but detected no significant OTUs (all adj $P \geq 0.075$).

Discussion

In this study, we sought to determine if the bacterial cecal microbiotas of TLGS were impacted by captive versus wild environments. We compared summer microbiotas between TLGS born in captivity that consumed a chow diet with wild-caught TLGS that consumed a natural, wild diet. We found that Wild microbiotas had greater richness and phylogenetic diversity and decreased variance in beta diversity compared to Captive microbiotas. Important taxa that contributed to differences in beta diversity and were core OTUs were primarily classified to the families *Lachnospiraceae* and *Ruminococcaceae*. We also described the microbiotas of captive TLGS across the year (summer, winter, and spring) using Illumina-based sequencing as all previous rodent hibernation microbiota studies to date have used either 454 pyrosequencing [56–59] or clone libraries [60].

Animals born and raised in the wild experience several environmental features that can affect their gut microbiota composition and therefore influence the host-microbe symbiosis. The composition and availability of food is arguably the most important environmental influence as these can affect early microbiota development and host-microbe relationships throughout life [12, 13, 16, 17]. Although commercial animal chow provides adequate macro- and micro-nutrients for generalized rodents, precise recapitulation of the wild diet is difficult, especially for omnivores like the TLGS. Foraging in the wild also provides opportunities for animals to ingest microbes that contribute to their gut microbiota. This variable is largely missing in captive diets, which can have significant effects on microbiota diversity and the host-microbe symbiosis [30].

Another potential variable is host age. Studying animals in captivity is useful because it provides precise age information that is often unavailable for wild animals. In our study, we were unable to assign exact ages to the Wild group; however, based on their body weights at capture (Table S1), we predicted that they were likely adults of at least 13 months of age. Therefore, the Wild squirrels were all older than the Captive squirrels. Previous TLGS microbiota studies have found that there are few differences between the summer microbiotas of wild-caught mothers and the summer microbiotas of their captive pups that were born and raised in captivity with known ages [56, 57]. Therefore, we posit that age differences between our Wild and Captive TLGS would not significantly bias our results.

Overall, we found that differences in the alpha diversity of content and mucosa microbiotas between the Captive and Wild groups were attributed to the Wild group's larger number of OTUs with low relative abundances. The Wild group had significantly more unique OTUs (Fig. 2A) and higher phylogenetic diversity (Fig. 2B) compared to Captive. These additional Wild OTUs were from numerous different taxa rather than from a single or closely related group of taxa as there was no difference in the average phylogenetic distance among all species pairs (Fig. 2C). Most of the Wild OTUs also had low relative abundances, demonstrated by the lack of difference in Shannon's index (Fig. 2D).

Beta diversity of content and mucosa microbiotas between Wild and Captive groups were different with respect to OTU identities, relative abundances, and phylogenetic relatedness (Fig. 3). Wild content microbiotas also had less variation than Captive content microbiotas; in mucosa microbiotas, this was true only in unweighted UniFrac. Because diet is a known driver of the microbiota, we expected the Wild

group to have higher variation compared to the Captive group; however, we found the opposite to be true. This has not been reported in other rodent studies, although that may be partially due to the scarce use of beta diversity variance tests in such studies [21, 23]. One possible explanation is that the ecological and evolutionary pressures of hosts living in the wild (e.g., more sporadic food availability, more energy expenditure to avoid predation) drive Wild microbiotas to converge and become similar to each other. In contrast, the different pressures in captivity (e.g., consistent food availability and lack of predators) [61] may cause variation in the microbiota to be less constrained.

Although Captive and Wild microbiotas had different alpha and beta diversities, we detected the same phyla in both groups, with no significant differences in relative abundances (Table 1); however, we did identify trends in the *Proteobacteria* and *Cyanobacteria* in content microbiotas. The *Proteobacteria* displayed a trend of higher relative abundance in Captive than in Wild microbiotas, which contrasted with results in other wild rodent studies where the *Proteobacteria* have higher relative abundances in wild compared to captive animals [6, 25, 62]. The *Cyanobacteria* displayed the opposite trend with higher relative abundance in Captive than in Wild microbiotas. All TLGS *Cyanobacteria* OTUs were classified to the class *Melainabacteria* and the order *Gastranaerophilales*. *Melainabacteria*, a proposed sister phylum to *Cyanobacteria*, is reported to not be autotrophic [63–65] and has been detected in diverse environments ranging from the human gut to groundwater [63].

We identified the families *Lachnospiraceae* and *Ruminococcaceae* as the predominant classifications of core OTUs in both Captive and Wild microbiotas (Tables S5 and S6) and of OTUs that explained Bray-Curtis dissimilarity (Table 2 and S4). There were more core OTUs in Wild compared to Captive microbiotas, which is not surprising given that Wild microbiotas had more unique OTUs. Just under half of the Captive core OTUs were also Wild core OTUs, demonstrating that there is reasonable overlap between the two groups despite their different alpha and beta diversities. Bray-Curtis dissimilarity between Captive and Wild microbiotas was explained by several *Lachnospiraceae* OTUs that had higher relative abundances in Wild than in Captive microbiotas, which is consistent with what has been observed in wild and captive mice [22, 24].

It is important to note that all rodent hibernation microbiota studies to date have used either 454 pyrosequencing [56–59] or clone libraries [60]. However, advances in sequencing technology have resulted in the vast majority of microbiota studies using Illumina-based sequencing. Because different sequencing technologies are known to introduce various biases [66–68], we considered if seasonal changes in the microbiotas of captive TLGS across the year (summer, winter, and spring) were consistent between Illumina sequencing and pyrosequencing. Overall, our findings from Illumina-based sequencing were generally consistent with those based on 454 pyrosequencing. We identified more phyla than in previous studies, but only found changes in the relative sequence abundances of the dominant phyla, and not in those with lower relative abundances [56, 57]. This suggests that trends in taxa with high relative abundances are conserved regardless of sequencing technology, but there may be differences in taxa with low relative abundances. We also confirmed previous findings that a small number of core OTUs persist in the TLGS cecal microbiota throughout the year despite seasonal shifts in the host's diet

[57]. Finally, our results with respect to alpha and beta diversity in winter (Torpor and IBA) and active groups (Summer and Spring) were similar, although we note that there were some minor differences in the gut microbiotas of active group squirrels. Taken together, these data confirm previous findings on the TLGS gut microbiota and address concerns that may arise due to biases inherent to the two sequencing technologies.

Conclusions

This is the first study to compare the microbiotas of captive and wild rodent hibernators. We demonstrated that Wild TLGS microbiotas have increased richness and phylogenetic diversity, and decreased variation in beta diversity compared to Captive TLGS microbiotas. In both Captive and Wild microbiotas, important taxa that are core OTUs or significantly contribute to beta diversity are predominantly in the families *Lachnospiraceae* and *Ruminococcaceae*. Given the significant differences between wild and captive TLGS microbiotas, it is important that future research determines how long it takes for wild microbiotas to change in captivity and what those changes entail. We also reported seasonal differences in captive TLGS microbiotas throughout the annual cycle by using Illumina-based 16S rRNA gene sequencing. Results about alpha diversity, beta diversity, and taxa with high relative abundances were consistent with past conclusions based on 454 pyrosequencing, but some differences emerged when comparing Summer and Spring seasons and in the analysis of low abundance taxa. Because next-generation sequencing continues to advance quickly, continued methodological comparisons should be conducted to evaluate any biases introduced by new methods. Taken together, our results help improve reproducibility and experimental design of future hibernation microbiota studies.

Abbreviations

ANOVA

analysis of variance

IBA

interbout arousal

MPD

mean pairwise distance

OTU

operational taxonomic unit

PBS

phosphate-buffered saline

PCoA

principal coordinate analysis

PCR

polymerase chain reaction

PERMANOVA

permutational multivariate analysis of variance

SIMPER

similarity percentages test

T_b

body temperature

TLGS

13-lined ground squirrel

Declarations

Ethics approval

All procedures were approved by the University of Wisconsin-Madison Institutional Animal Care and Use Committee under protocols V001229 and V005481.

Consent for publication

Not applicable.

Availability of data and material

All FAST Q files were submitted to the NCBI's Short Read Archive and are publicly available under bioproject number PRJNA742778 (<https://www.ncbi.nlm.nih.gov/bioproject/PRJNA742778>). Mothur logfiles and output files, as well as all R code used to perform statistical analyses and generate figures, are publicly available at <https://github.com/ednachiang/CaptiveWild>.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

EC, HVC, and GS designed the study. EC and HVC collected samples. EC and CLD prepared and sequenced samples. EC analyzed the data. EC wrote the manuscript with help from CLD, HVC, and GS.

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Figures

Figure 1

Experimental design. Cecal content and mucosa were collected from summer captive and wild TLGS, as well as captive TLGS across the year. The black box indicates the two summer groups that were used in the comparison of Captive and Wild microbiotas. For the description of captive TLGS across seasons, four groups were used: Summer, Torpor, IBA, and Spring. Summer and Spring groups represent active seasons, while Torpor and IBA groups represent the winter season.

Figure 2

Alpha diversity of summer Captive and Wild content microbiotas. Violin plots display four alpha diversity metrics: (A) the number of unique OTUs, (B) Faith's phylogenetic diversity, (C) phylogenetic evenness (MPD), and (D) Shannon's diversity. An asterisk indicates a significant difference (adj P < 0.05) and "ns" indicates no significant difference (adj P > 0.05).

Figure 3

Beta diversity of summer Captive and Wild content microbiotas. (A) Weighted UniFrac, (B) unweighted UniFrac, and (C) Bray-Curtis dissimilarity are displayed on PCoA ordinations. Groups are depicted with different colors.

Figure 4

Alpha diversity comparison among content microbiotas of captive groups. Violin plots display four alpha diversity metrics: (A) the number of unique OTUs, (B) Faith's phylogenetic diversity, (C) phylogenetic evenness (MPD), and (D) Shannon's diversity. Groups that share a letter are not significantly different (adj $P > 0.05$), whereas groups that share no letters are significant different (adj $P < 0.05$). Metrics with no significant comparisons between groups are indicated with "ns."

Figure 5

Beta diversity of content microbiotas from captive groups. (A) Weighted UniFrac, (B) unweighted UniFrac, and (C) Bray-Curtis dissimilarity are displayed on PCoA ordinations. Groups are depicted with different colors.

Supplementary Files

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