COVER SHEET

TITLE: Functional Changes in gut microbiota across the hibernation cycle of Ictidomys tridecemlineatus examined by stable isotope-assisted labeling

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ABSTRACT

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The hibernation cycle of the 13-lined ground squirrel, which includes fasting during winter months, drives changes in taxonomic composition of the gut microbiota. This study employs stable isotope-assisted labeling (SIAL) as a novel approach to investigate, *in vivo*, functional capacities of changing microbiotas across the hibernation cycle. Capacity to degrade ^{13}C-inulin and ^{13}C-mannitol, as indicated by change in breath $\delta^{13}CO_2$ after oral gavage, was greater in summer than winter. Prior antibiotic (ABX) treatment reduced $\delta^{13}CO_2$ in summer after ^{13}C-inulin or ^{13}C-mannitol gavage. Results suggest that SIAL effectively tracks seasonal changes in functional capacity of the microbiota to degrade substrates, and that ABX treatment depletes bacterial populations as indicated by significant reductions in $\delta^{13}CO_2$. This has validated SIAL as a technology to determine the functional ability of a microbiota to degrade various substrates.

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Abstract

The hibernation cycle of the 13-lined ground squirrel, which includes fasting during winter months, drives changes in taxonomic composition of the gut microbiota. This study employs stable isotope-assisted labeling (SIAL) as a novel approach to investigate, *in vivo*, functional capacities of changing microbiotas across the hibernation cycle. Capacity to degrade ^{13}C-inulin and ^{13}C-mannitol, as indicated by change in breath $\delta^{13}CO_2$ after oral gavage, was greater in summer than winter. Prior antibiotic (ABX) treatment reduced $\delta^{13}CO_2$ in summer after ^{13}C-inulin or ^{13}C-mannitol gavage. Results suggest that SIAL effectively tracks seasonal changes in functional capacity of the microbiota to degrade substrates, and that ABX treatment depletes bacterial populations as indicated by significant reductions in $\delta^{13}CO_2$. This has validated SIAL as a technology to determine the functional ability of a microbiota to degrade various substrates.

Introduction

Like other hibernating animals, the 13-lined ground squirrel (*Ictidomys tridecemlineatus*) follows an annual metabolic cycle in which rapid energy storage in adipose tissue during summer months provides sustainable energy throughout the depressed metabolic states of torpor. However, torpor is not a static state of constant metabolic depression. Hibernating squirrels experience interbout arousals, which are brief periods (~12 hours) in hibernation in which the squirrel returns to a normal metabolic rate and body temperature. Studying the symbiosis between the ground squirrel and the gut microbiota is advantageous because the host animal naturally goes through periods of extended fasting, allowing for the unique opportunity to explore the effect of dietary substrate availability on the gut microbiota, without posing a health risk to the host animal (2). This is particularly relevant now that there is overwhelming evidence that dietary abundance and composition drives the structure of gut microbial communities (8).

Indeed, recent studies suggest the gut microbiota of the 13-lined ground squirrel changes throughout the hibernation cycle, most notably resulting in shifts in the dominant phyla of the mammalian gut microbiota, Bacteroidetes and Firmicutes (3,4,5). The relative abundance of Firmicutes in summer microbiotas, when squirrels are ingesting diets with high plant content, is greater than in winter (3). For example, there is a larger abundance of the family *Lachnospiracea*, which contain many plant-degrading species, in summer microbiotas relative to winter (3). In contrast, winter microbiotas contain a greater abundance of the phylum Bacteroidetes, including the endogenous host mucin-degrading bacteria *Akkermansia muciniphila* (3). While approaches used in previous studies provide information on compositional changes of the gut microbiota in the hibernation cycle, these studies are limited

in the conclusions drawn on the functional significance of these shifts and their impact on the host squirrel.

This study aimed to fill this gap by examining functional significance of seasonal changes in the gut microbiota of squirrels through the novel application of stable isotope assisted labeling (SIAL). This is achieved by oral administration of ^{13}C labeled substrates that cannot be metabolized by mammalian enzymes but can be degraded by bacterial enzymes followed by measurement of the ratio of $^{13}C/^{12}C$ in expired CO_2 ($\delta^{13}C$) (1).

Breath responses to two ^{13}C-labeled substrates were examined. Inulin, a complex plant glycan, was used to examine the ability of the gut microbes to degrade typical dietary substrates ingested in the non-hibernating months. We predicted that $\delta^{13}C$ breath response to inulin would decrease from summer to winter, reflecting the fall in functional capacity of the gut microbiota to fully degrade plant carbohydrates. In contrast, we predicted $\delta^{13}C$ breath response to mannitol, a simple sugar alcohol, would be maintained in the hibernation microbiota similar to summer responses, or show less of a reduction compared with winter responses to inulin.

To further demonstrate the microbial origin of changes in $\delta^{13}C$ after inulin or mannitol gavage, we depleted the microbiota of a subset of squirrels in summer and winter with a broad spectrum antibiotic cocktail (ABX). Thus, we hypothesized that changes in breath $\delta^{13}C$ breath response to substrate administration would be reduced in ABX treated squirrels regardless of season.

Methods

Animal Care

Pregnant ground squirrels were collected from the wild in May 2017 in the Madison area. Squirrels from their litters became the animals used in breath experiments. They remained at Charmany Instructional Facility eating a prescribed rat chow diet in a room held at 22°C and 12L:12D light cycle. In mid-September, remaining squirrels were moved to a room kept at 4°C and complete darkness except brief (<10 minute) periods of low light for health and activity checks. Once regular torpor bouts were established, food and water were removed from animal cages. Winter squirrels received daily health checks while in the cold room. Squirrels were used after 3-4 months. On the day of breath experiments, prior to gavage and breath collection, hibernating squirrels were moved back to laboratory room temperature (~22°C) and were allowed to arouse naturally, as indicated by return to euthermia and increased physical activity.

ABX Treatment

A subset of squirrels from both mannitol and inulin treatment groups in summer and winter were treated with a broad-spectrum antibiotic cocktail (ABX) that contained ampicillin (1 g/L), vancomycin (0.5 g/L), neomycin (1 g/L), and metronidazole (1 g/L) administered in drinking water. For summer squirrels, ABX was administered for 2 weeks prior to the breath experiment. In winter squirrels, ABX was administered for 2 weeks prior to their entrance into the 4°C cold room. Because the hibernating animals were kept in the cold room to hibernate for several months prior to the experimentation date and during this time had no water intake, hibernating squirrels were orally gavaged with 1 mL of the ABX solution 3 times during hibernation. These additional gavage dosages of ABX were intended

to serve as a booster to ensure continual bacterial depletion in the gut of the hibernating ground squirrels.

SIAL Breath Experiments

^{13}C-inulin (500 mg/kg) or ^{13}C-mannitol (150 mg/kg) were administered to summer and winter squirrels via oral gavage. Pilot studies showed that intraperitoneal administration of substrates resulted in little or no increase in measured $\delta^{13}C$ in breath, where an increase in $\delta^{13}C$ was observed when the compounds were administered directly to the GI tract via gavage. These pilots demonstrated that $\delta^{13}C$ measured using this technique is a direct result of microbial degradation of administered substrates and not a result of degradation by mammalian enzymes.

After gavage, squirrels were placed in a chamber to collect expired CO_2. A cavity ring-down spectroscopy (CRDS) machine obtained breath samples approximately every 15 minutes and using an internal standard, calculated $\delta^{13}C$ for each sample. Squirrel breath samples were collected for approximately 1 hour prior to gavage to establish baseline $\delta^{13}C$ values. After gavage, breath was collected for 2.5 additional hours for ^{13}C-mannitol and 4.5 hours for squirrels treated with ^{13}C-inulin. After breath measurements, squirrels were euthanized, and gut contents and tissues were harvested for future experiments.

qPCR for Bacterial Abundance Assessment

Bacterial DNA in cecum content samples was quantified to estimate total bacterial abundance. Bacterial DNA was extracted from cecum content collected from euthanized squirrels after the breath experiments. DNA concentrations were measured using Quant-iT. Due to decreased bacterial DNA in cecum content of ABX treated squirrels, available bacterial DNA stocks measured 0 ng/µl. Control squirrel cecum content bacterial DNA extracts had non-zero values, and these bacterial DNA stocks were diluted to 10 ng/µl.

Regardless of stock concentration, 2.5 µl of stock was added to reactions. Extracted DNA from summer control and ABX-treated squirrels gavaged with only saline was added to a master mix containing SYBR Green Reagent and forward and reverse universal bacterial primers. A qPCR reaction was performed with these experimental samples and samples of DNA extracted from *Akkermansia muciniphila* that was previously purified from 13-lined ground squirrel cecal contents. The *A. muciniphila* qPCR results were used to construct a standard curve, which yielded an equation from which the quantity of DNA in experimental samples was calculated.

Statistics

Sample sizes ranged from 3-6 squirrels per experimental group and are indicated in figure legends. Peak breath response was analyzed by comparing mean changes in $\delta^{13}C$, quantified by subtracting baseline $\delta^{13}C$ responses prior to gavage from peak $\delta^{13}C$ observed after gavage for each squirrel. Statistical significance was assessed using 2-way ANOVA with post-hoc Tukey tests ($P \leq 0.05$).

Results

Baseline $\delta^{13}C$

Baseline $\delta^{13}C$ measured before administration of ^{13}C-inulin or ^{13}C-mannitol were greater (i.e., less negative) in summer squirrels relative to winter hibernating squirrels (Fig. 1). There were no differences between baseline $\delta^{13}C$ responses in control (Con) or ABX treated animals within each seasonal group for either substrate.

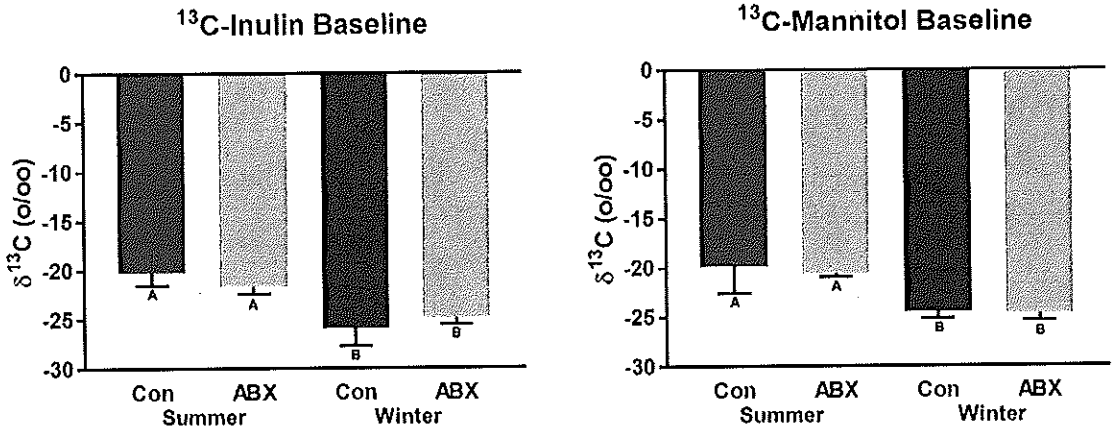

Figure 1. Baseline $\delta^{13}C$ prior to inulin or mannitol gavage. Bars show means ± SD. Groups with different letters are significantly different, $P<0.05$. Sample sizes for inulin are N=5 for all groups except Summer ABX (N=3; one outlier was removed). For mannitol, Summer: Con (N=5), ABX (N=4); Winter: Con (N=6), ABX (N=5).

Breath Response to ^{13}C-inulin

Gavage of ^{13}C-inulin to control squirrels induced a change in breath $\delta^{13}C$ in both seasons (Fig. 2). However, the response was lower in the winter squirrels (Fig. 2). In summer squirrels, pretreatment with ABX reduced the $\delta^{13}C$ response to ^{13}C-inulin relative to untreated controls. A similar trend was observed in winter squirrels, although the effect was not significant.

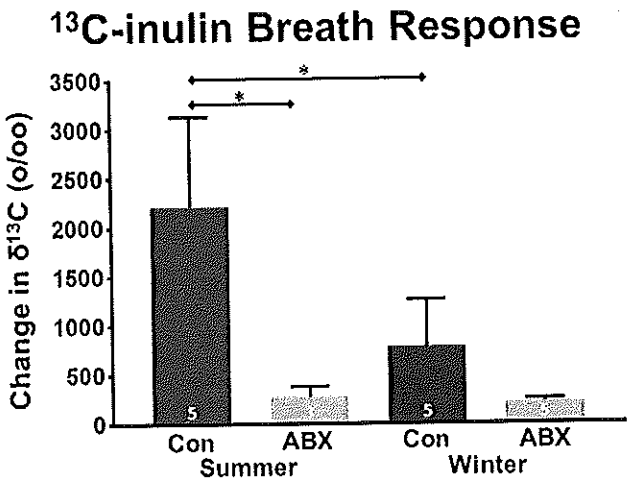

Figure 2. Change in breath $\delta^{13}C$ after gavage with ^{13}C-inulin. Bars show means ± SD. *, groups significantly different ($P<0.05$). Sample sizes are shown in Fig. 1 legend.

One outlier was excluded from the summer inulin ABX group. This animal displayed an atypically large $\delta^{13}C$ response compared with other ABX-treated squirrels, and analysis of cecum content DNA yield revealed a higher than normal DNA concentration for this squirrel's microbiota (56.8 ng DNA/mg cecum content vs. 0 ng DNA/mg cecum content). Based on these observations we concluded that the ABX treatment was not effective in depleting the gut microbiota of this squirrel to a level sufficient to be included with the other ABX-treated squirrels in the data set.

Breath Response to ^{13}C-mannitol

Gavage of ^{13}C-mannitol to control squirrels induced a change in breath $\delta^{13}C$ in both seasons (Fig. 3). As for the response to ^{13}C-inulin, the $\delta^{13}C$ response to ^{13}C-mannitol was reduced in the winter squirrels relative to summer. Pretreatment with ABX reduced the $\delta^{13}C$ response to ^{13}C-mannitol in summer squirrels compared with untreated controls, but had no effect in the winter animals (Fig. 3).

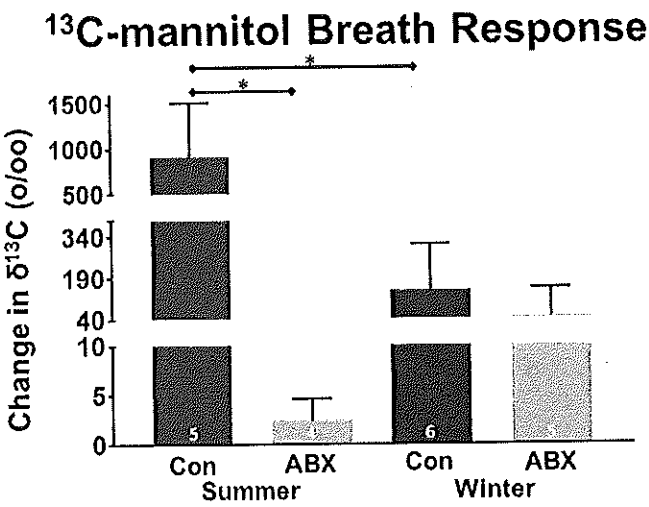

Figure 3. Change in breath $\delta^{13}C$ after gavage with ^{13}C-mannitol. Bars show means ± SD. *, groups significantly different ($P<0.05$). Sample sizes are shown in Fig. 1 legend.

One squirrel in the winter ABX group had an atypically large $\delta^{13}C$ response compared with other ABX-treated winter squirrels gavaged with ^{13}C-mannitol. Cecum content DNA yields have not yet been measured for any winter squirrels in our data set, making it difficult to determine whether there was inadequate microbial depletion by ABX in this particular squirrel. Therefore, this squirrel was retained in the data set. If, as expected, this squirrel has an atypically high cecum content DNA yield, it will be excluded from further analysis.

qPCR Validation of ABX Microbial Depletion

qPCR with a universal bacterial primer was used to verify efficacy of ABX treatment in depletion of the gut microbiota. This analysis examined bacterial DNA extracted from cecum contents of summer squirrels that received a saline gavage in their breath experiments. There was a decrease in the amount of bacterial DNA amplified in summer saline ABX-treated squirrels (0.1 ng ± 0.09, N=4) relative to summer saline untreated squirrels (28.5 ng ± 5.1, N=4).

Discussion

Baseline Breath Response

Prior to administration of either ^{13}C-labeled substrate, baseline $\delta^{13}C$ was lower (i.e., more negative) in all winter squirrels relative to summer squirrels. This is consistent with the metabolic physiology of seasonal hibernators, because during the winter fast the metabolism of hibernating squirrels shifts to utilize primarily stored lipids to fuel torpor periods and especially interbout arousals. The lower $\delta^{13}C$ values in the hibernators indicate increased lipid metabolism, whereas the higher $\delta^{13}C$ values in summer squirrels suggest mixed carbohydrate and lipid metabolism (9). Furthermore, these results show no change in $\delta^{13}C$ between control and ABX squirrels in either season. This demonstrates that ABX treatment does not significantly alter basal metabolism as reflected in no change in baseline $\delta^{13}C$ relative to untreated control squirrels.

Seasonal Effects on Mannitol and Inulin Degradation

Analysis of seasonal differences in $\delta^{13}C$ breath responses provided insight into the differential degradation capacities of the gut microbiota across the hibernation cycle. Breath experiments demonstrated a decrease in $\delta^{13}C$ after inulin gavage, which indicates decreased capacity to degrade inulin by the winter squirrel gut microbiota relative to the summer gut microbiota. Reduced capacity to degrade a complex plant glycan is consistent with previous studies that demonstrated taxonomic shifts in the squirrel gut microbiota. For example, relative abundances of microbial taxa known to degrade plant substrates, such as *Lachnospiracea*, are reduced in winter relative to summer (3). The observed changes in functional capabilities to degrade plant substrates such as the complex glycan inulin has potential implications for the structure of the gut microbial community and the impacts of these shifts on the squirrel host. Reduced substrate availability for the plant-degrading

microbiota is a potential driving force to decrease abundance of these taxa by eliminating their metabolic niche from the gut. Reduced presence of dietary substrate degrading microbes may allow for an increase in microbes that are able to degrade substrates more available in the winter gut, such as host-derived mucins. This could ultimately provide some benefit to the host squirrel in hibernation, as the microbes that remain at high numbers in the winter can metabolize endogenous substrates, producing metabolites such as short-chain fatty acids that can be absorbed and utilized for energy by the host animal.

The capacity of the squirrel gut microbiota to degrade mannitol was also reduced in winter relative to summer. We originally hypothesized that the capacity of the gut microbiota to degrade mannitol would show less of a decrease across seasons, in part due to its simple structure, relative to inulin, theoretically allowing it to be more easily metabolized. However, decreased capability of degrading mannitol in the winter matched the trend observed with inulin. This suggests that while mannitol is a less complex substrate, the microbial populations capable of its metabolism are also reduced in relative abundance in the winter relative to summer such that the degradation capacity for mannitol is reduced significantly. A decreased role of mannitol-degrading microbes in the squirrel gut in hibernation could be a result of decreased availability of substrates for mannitol degrading bacteria from diet, thus reducing their ability to thrive in the gut community.

Comparing known taxonomic information with the functional capabilities assessed in breath experiments establishes a more cohesive understanding of how the gut microbiota community structure changes throughout the hibernation cycle. These new findings on microbiota capacity to degrade substrates allows for the understanding of the implications these taxonomic changes have for the host in terms of availability of the microbial-degraded products. As a whole, these breath experiments identified a reduced capacity of the squirrel gut microbiota to degrade both mannitol and inulin in winter relative to summer, likely driven

by the changing dietary demands of the squirrel host influencing the ability of mannitol- and inulin-degrading microbes to thrive.

ABX Reduction of Breath Responses

The breath experiments verified that ABX depletion of the gut microbiota decreased its capacity to degrade inulin and mannitol in summer. This result was expected as the ABX treatment was designed to deplete the gut microbial abundance in treated squirrels relative to untreated squirrels. There was a similar decrease in capability to degrade mannitol after ABX treatment in summer.

No difference in $\delta^{13}C$ breath response was observed between control and ABX in winter after inulin or mannitol gavage. A potential reason for this could be the already reduced $\delta^{13}C$ responses in winter control groups for both substrates making further decreases in $\delta^{13}C$ in the ABX groups less significant. Furthermore, there was considerable variation in the winter control groups relative to their mean change in $\delta^{13}C$ values.

The decrease in degradation capacity for inulin and mannitol in summer after ABX treatment indicates success of the ABX treatment in decreasing populations of microbes which are capable of degrading inulin and mannitol respectively. Furthermore, a reduction in breath $\delta^{13}C$ responses of squirrels with ABX-depleted gut microbiota in summer validates the application of SIAL technology coupled with breath testing to assess changes in microbial activity. Thus, SIAL technology can be applied to gain insight not only on the metabolic capabilities of the squirrel gut microbiota across the hibernation cycle, but potentially investigating functional capacity of the microbiota in any system of interest.

ABX Depletion of the Gut Microbiota

The quantity of bacterial DNA amplified from DNA extracts of cecum content in summer animals gavaged with saline indicated a reduction in overall bacterial DNA

abundance in squirrels treated with ABX. This supports that the current ABX treatment used is effective in depleting the gut microbiota in treated squirrels. Coupling this evidence for bacterial DNA depletion with the observed reduction breath $\delta^{13}C$ response for ABX-treated squirrels provides validation that this antibiotic treatment is effective at reducing both the abundance of microbes present in the gut and the capability of the squirrel gut microbiota to degrade ^{13}C-inulin and ^{13}C-mannitol.

Conclusions/Future Directions

This study utilized novel approaches to examine the functional implications of gut microbial changes during the hibernation cycles of squirrels *in vivo.* Results of the inulin breath studies allow us to conclude that the squirrel gut microbiota capacity to degrade inulin decreases in winter compared to summer. This finding confirmed our hypothesis that shifts in microbial taxa composing the gut microbiota of squirrels from summer to winter reflects a reduced ability of the winter microbiota present to degrade dietary substrates. Contrary to our hypothesis, our results indicated reduced ability of the squirrel gut microbiota to degrade mannitol in winter relative to summer, rejecting the hypothesis that this simpler sugar alcohol would be equally metabolizable in summer and winter, or show only a modest difference between the two seasons. This suggests that specific microbial taxa associated with mannitol metabolism may decrease in relative abundance from summer to winter. Our results also confirmed that ABX treatment depleted the gut microbiota as indicated by decreased degradation of inulin and mannitol in summer.

In a broader sense, these experiments have validated SIAL as a valuable technology to measure changes in the ability of a microbial community to degrade various substrates. At a time when many microbiome studies focus on surveying the composition of communities, SIAL technology provides the ability to perform more hypothesis-driven work that assesses

real time, *in vivo* functional capacity. This technology allows for future studies on gut microbe changes in hibernating squirrels, but also has potential to be used in other study systems. Functional gut microbial studies using this methodology could improve understanding of gut microbial community disturbance, such as those which occur in human patients receiving total parenteral nutrients (TPN). This is a therapy in which necessary nutrients are delivered intravenously, as a result of various GI conditions in which nutrients cannot be taken in through the GI tract. TPN is known to induce gut microbe composition changes believed to cause problems with gut permeability and inflammation in mice (6). Technologies such as SIAL could be applied in this situation to better understand the specific functional changes caused by the resulting microbial community after this therapy, which may provide insight into prevention of the aforementioned negative side effects.

Experiments concurrent with the breath experiments will provide greater insight into the physiological implications of shifts in the gut microbiota of the ground squirrel across the hibernation cycle. 16S sequencing after experiments are completed identifies specific taxonomic compositions of the gut microbiota in ground squirrels subjected to the breath experiments. Furthermore, metabolomics approaches will allow for the determination of the fates of ^{13}C liberated from substrates that was not expired in breath, but rather incorporated into metabolites and transported to various ground squirrel tissues (i.e., the metabolome). Combining information on functional ability to degrade substrates, taxonomic composition of gut microbiomes, and distribution of metabolized substrates will provide a clear and comprehensive picture of the complex impacts of hibernation on both the gut microbial populations as well as the host squirrel's physiology. Future studies beyond these current experiments could involve the examination of additional substrates in breath experiments. In particular, the use of substrates which are favored by taxa known to increase in relative abundance in winter, such as mucin glycoproteins, could provide insight into potential

beneficial effects of these taxonomic shifts which may be protective of the host's extreme gastrointestinal state during hibernation.

To conclude, SIAL serves as a useful technique to assess whether the capacities of the ground squirrel gut microbiota to degrade inulin and mannitol are reduced during winter hibernation relative to the fed, summer season. Our findings provide a functional context and significance for previous studies identifying shifts in microbial taxa with presumed differences in metabolic capabilities. Further experimentation will provide more insight into the exact community compositions studied here, as well as the metabolic fate of ^{13}C integrated into the host squirrel metabolome. These findings foster understanding of how the gut microbiota of the ground squirrel adapts to extreme seasonal changes in physiology in order to maintain a microbial community which maximizes benefit to the host's needs across seasons. The novel experimental techniques verified in this study have the potential to revolutionize *in vivo* study of microbial communities through critical understanding of functional significance of microbial shifts.

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