



# Changes in the Intestinal Microbes of Wild *Spermophilus dauricus* (Rodentia: Sciuridae) in Different Periods of Hibernation (A Model of Gut Ischemic Reperfusion Injury)

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## ABSTRACT

Reperfusion injury after intestinal ischemia and hypoxia is a complex and comprehensive disease with a poor prognosis and high fatality rate. However, whether it can promote or slow down intestinal ischemia-reperfusion injury remains unclear. *Spermophilus dauricus* is a hibernation animal with a short awake time that interrupts the long hibernation period. When these events occurred, the long-term ischemic intestine in hibernation rapidly congested and entered the hibernation and ischemic state again. However, *Spermophilus dauricus* is not sick, and this might be a good model for gut ischemic reperfusion injury. In this animal research study, 16s rRNA gene sequencing was used to detect the bacterial community composition of *S. dauricus* intestine before, during, and after the artificial hibernation process and short awakening hibernation, respectively. The results showed that there was no significant alpha diversity during the four periods; however, the beta diversity was different, especially in the awakening period. These differences in beta diversity may be due to the significantly increased abundance of 11 kinds of bacteria, including *Anoxybacillus*, *Geobacillus*, *Klebsiella*, *Thermus*, and *Acinetobacter* ( $P < 0.05$ ), and the significantly reduced abundance of 16 species of bacteria, including *Acinetobacter*, *Aquabacterium*, *Acidovorax*, *Thermus*, and *Klebsiella* ( $P < 0.05$ ) in the awakening period. This study provides guidance and reference for the exploration of animal hibernation mechanisms and research on intestinal ischemic re-enema injury.

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Conceptualization, CS and JL. Methodology, CS. Formal analysis, CS and JG. Investigation, CS, ML and QY. Resources CS. Data curation, CS. Writing original draft preparation, CS. Writing review and editing, CS. Supervision, CS. Project administration, CS and JL. Funding acquisition, CS and JL. All authors have read and agreed to the published version of the manuscript.

## Key words

Hibernation, Awakening period of hibernation, Gut microbes, Intestinal ischemic re-enema injury, 16s rDNA

## INTRODUCTION

Ischemic injury is an organic injury to the organs of body in a state of ischemia and hypoxia (Hummitzsch *et al.*, 2019). After the tissue cells have suffered ischemia for a certain period of time, blood flow is restored and the degree of tissue damage increases sharply. Reperfusion injury after intestinal ischemia and hypoxia is a complex and comprehensive disease with a poor prognosis and high fatality rate. It is important to study the mechanism of intestinal ischemia-reperfusion injury to prevent intestinal injury (Ikebuaso *et al.*, 2011).

In recent years, an increasing number of studies have

revealed that gut microbes play an important regulatory role, and they can perform a function as the second gene pool in organisms (Barko *et al.*, 2017). Bile acids produced by intestinal microbes can regulate intestinal regulatory T cell population and affect immunity (Liu *et al.*, 2020). The positive or negative correlation between the intestinal microbial community and insulin resistance can affect plasma metabolism and immune performance of the body (Fujisaka *et al.*, 2018). Intestinal microbes can regulate glucose homeostasis and energy balance via the intestinal-brain axis (Kim *et al.*, 2018; Bauer *et al.*, 2018). Moreover, the composition of fungal flora can slow down the injury of intestinal ischemia and reperfusion (Papoff *et al.*, 2012). Colonic mucus in the intestine can cause intestinal bacteria to interact with colon cells, which is related to inflammation. Excretion can promote the recovery of mucus after intestinal ischemia and slow reperfusion (Grootjans *et al.*, 2013). Short-chain fatty acids produced by microorganisms in the intestine can inhibit histone deacetylase activity and expression during chromosome reorganization, thereby improving the intestinal tract ischemic reperfusion injury (Andrade-Oliveira *et al.*, 2015). In surgical liver surgery, local

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congestion of the intestine and colonization of bacteria in the portal vein of the intestine or through the intestine-liver axis are regulated via the lipopolysaccharide (LPS)-toll-like receptor 4 (TLR4) pathway. TLR4 in the liver causes cancer cell regeneration (Orci *et al.*, 2018). Studies have shown that symbiosis of intestinal microbes can regulate the intestinal barrier through pregnane X receptor (PXR) and TLR4 (Madhukumar *et al.*, 2014). They can also affect TLR2, TLR4, and its adaptor protein, myeloid differentiation primary-response 88 (MyD88), oxidative stress, nitric oxide, or the formation of intestinal ischemic reperfusion injury (Yuji *et al.*, 2018). However, whether changes in the composition of intestinal bacterial flora and metabolites have a direct regulatory effect on intestinal ischemic reperfusion injury and whether they can promote or slow down intestinal ischemic reperfusion injury remains elusive.

This study used wild *S. dauricus* during hibernation as a research model. *Spermophilus dauricus* is a hibernation animal with a short awake time that interrupts the long hibernation period. When these events occur, the long-term ischemic intestine in hibernation rapidly congests and the animal enters the hibernation and ischemic state again. However, as *S. dauricus* is not sick, it might be a good model for gut ischemic reperfusion injury. During hibernation, the basal metabolism of *S. dauricus* is extremely low and the intestine is in a certain ischemic state. However, during the short awakening period, the physiological metabolism of *S. dauricus* rapidly recovers and blood reperfusion occurs. In this study, we selected the pre-hibernation, hibernation, post-hibernation, and awakening periods in the hibernation process as time nodes to explore the hibernation and awakening periods of *S. dauricus* (intestinal ischemic reperfusion period) microbial composition. We analyzed the physiological mechanism of *S. dauricus* against intestinal ischemic reperfusion from the perspective of microorganisms, studied the possible microbial regulation mechanisms related to the formation of intestinal ischemic reperfusion, and provided references for future research on intestinal ischemia-reperfusion injury.

## MATERIALS AND METHODS

### *Animal management*

All animal procedures (care, handling, and experimentation) followed the guidelines established by the rules for the administration of affairs concerning experimental animals. For studying gut microbes, 60 wild *Spermophilus dauricus* were divided into four groups, pre-hibernation (PRE, n= 15), middle hibernation group (HIB, n= 15, after 60 days of hibernation), intervene arousal

group (IBA, n=15), and post-sleep group (POST, n=15, 90±12 days after hibernation, 2 days after hibernation). Temperature and light were artificially controlled to simulate winter to facilitate natural entry of these species into the hibernation state. At the same time, the weather conditions were controlled to make *S. dauricus* appear in an arousal state according to their habits. Finally, three *S. dauricus* were randomly selected from each group for collection of intestinal feces because *S. dauricus* is a nationally protected animal in China. The digestive tract of the hibernating group was in a state of ischemia whereas that of the arousal group was in a state of ischemia reperfusion.

### *DNA extraction, PCR, and sequencing*

The total genomic DNA from samples was extracted using the E.Z.N.A.® feces DNA kit (Omega Bio-tek, Norcross, GA, USA) according to manufacturer's protocol. The primer set 515F-GTGCCAGCMGCCGCGGTAA-806R-GGACTACHVGGGTWTCTAAT was used for 16S rRNA gene amplification with specific barcodes. All PCR reactions were performed in 30 µL volume with 15 µL of Phusion® High-Fidelity PCR Master Mix (New England Biolabs), 0.2 µM of forward and reverse primers, and approximately 10 ng template DNA. Thermal cycling consisted of initial denaturation at 98°C for 1 min, followed by 30 cycles of denaturation at 98°C for 10 s, annealing at 50°C for 30 s, elongation at 72°C for 60 s, and finally, elongation at 72°C for 5 min. Products mixed at equidensity ratios were purified using the GeneJET Gel Extraction Kit (Thermo Fisher Scientific). All library amplifications were generated using the NEB Next® Ultra™ DNA Library Prep Kit for Illumina (NEB, USA). Finally, the library was sequenced on an Illumina MiSeq platform, and 300 bp paired-end reads were generated at BGI Tech Co., Ltd. (Shenzhen, China).

### *Sequence denoising, OTU (operational taxonomic units) clustering, diversity analysis, and statistics*

Raw reads were combined, trimmed, and dereplicated using Vsearch (v2.17.0) (Rognes *et al.*, 2016) to remove low-quality and mismatched reads. The high-quality reads were clustered into OTUs using Usearch v10.0.240 (Edgar and Valencia, 2018; Edgar, 2010). Reference-based chimeras were detected and removed using Vsearch, based on RDP\_6s\_V16\_sp (<http://rdp.cme.msu.edu/>). Following this, the feature table was prepared, and plastids and non-bacteria were removed using Vsearch (cutoff= 0.6). The lowest sequence number across all samples was used to rarefy each sample and equalize the sequencing depth. The rarefied sequences were calculated for alpha and beta diversity indices using Usearch. The OTUs were

annotated using GreenGene (v13.8, [ftp://greengenes.microbio.me/greengenes\\_release](ftp://greengenes.microbio.me/greengenes_release)). OTUs with a selected relative abundance of more than 0.2% were aligned using MUSCLE (Robert, 2004) to build a cladogram in iQtree (Lam-Tung *et al.*, 2015). The cladogram was visualized using table2itol (<https://github.com/mgoeker/table2itol>) and iTOL (<https://itol.embl.de/>). Biomarkers were predicted using LEfSe (Segata *et al.*, 2011) (<http://www.ehbio.com/ImageGP/index.php/Home/Index/index.html>). Finally, differences in bacterial abundance were analyzed using STAMP (v2.1.3, <https://github.com/dparks1134/STAMP>, Parks *et al.*, 2014). The two groups were compared using Welch's t-test, and P-value was adjusted using Benjamin-Hochberg FDR.

The data that support the study findings have been deposited into the CNGB Sequence Archive (CNSA) of the China National GeneBank BataBase (CNGBdb) with accession number CNP0002091.

## RESULTS

### Sample sequencing data statistics

The results showed that more than 31.34 Mbp raw data was present in each sample. The raw reads were greater than  $63373 \times 2$ . After cutting the barcode and primers, the availability of clean data was found to be greater than 95.16% in each sample. All tag numbers exceeded 47502 (Table I).

As the sequencing depth increased, the number of OTUs obtained from the sample saturated when it was above 20% in all groups (Fig. 1). Based on de-redundancy,

bacteria in HIB, IBA, POST, and PRE groups clustered into 44, 17, 43, and 65 effective OTUs, respectively. Three identical OTUs were present in the four groups and no unique OTUs were present in the IBA group; all OTUs were identical in the other three groups and only the abundance was different (Fig. 2).

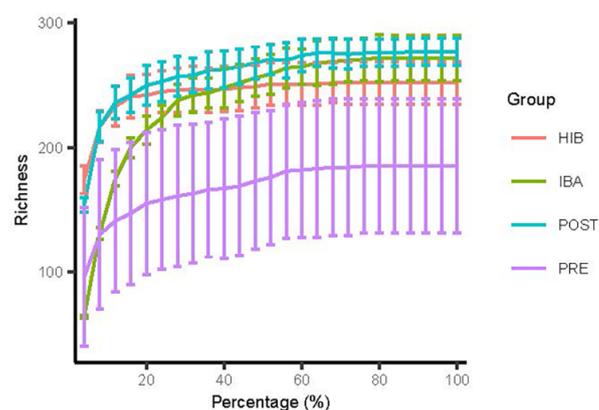


Fig. 1. The rarefaction curves of all groups.

### Alpha and beta diversities

After diversity analysis, no significant difference was observed in ACE, chao1, in vsimpson, richness, Shannon, and Simpson alpha diversity indices between each group (Fig. 3). No significant difference was observed in the abundance and type of bacteria in each group, and the distribution of bacteria in each group was uniform.

Table I. Sample sequencing data statistics.

| Sample name | Reads length (bp) | Raw data (Mbp) | Adapter (%) | N base (%) | Ploy base (%) | Low quality (%) | Clean data (Mbp) | Data utilization ratio (%) | Raw reads | Clean reads | Read utilization ratio (%) | Tag number |
|-------------|-------------------|----------------|-------------|------------|---------------|-----------------|------------------|----------------------------|-----------|-------------|----------------------------|------------|
| HIB1        | 250:250           | 31.69          | 0           | 0.07       | 0.002         | 1.752           | 30.58            | 96.51                      | 63373×2   | 61331×2     | 96.78                      | 52538      |
| HIB2        | 250:250           | 31.89          | 0           | 0.063      | 0.008         | 1.812           | 30.76            | 96.47                      | 63771×2   | 61687×2     | 96.73                      | 52280      |
| HIB3        | 250:250           | 31.34          | 0           | 0.059      | 0.002         | 1.571           | 30.35            | 96.84                      | 62682×2   | 60868×2     | 97.11                      | 51896      |
| IBA1        | 250:250           | 31.59          | 0           | 0.064      | 0.02          | 2.157           | 30.59            | 96.83                      | 63181×2   | 61246×2     | 96.94                      | 58503      |
| IBA2        | 250:250           | 31.2           | 0           | 0.068      | 0.021         | 2.113           | 30.21            | 96.82                      | 62406×2   | 60485×2     | 96.92                      | 58722      |
| IBA3        | 250:250           | 31.57          | 0           | 0.063      | 0.03          | 2.834           | 30.32            | 96.04                      | 63140×2   | 60701×2     | 96.14                      | 58630      |
| POST1       | 250:250           | 31.36          | 0           | 0.052      | 0             | 1.585           | 30.37            | 96.85                      | 62714×2   | 60900×2     | 97.11                      | 52477      |
| POST2       | 250:250           | 31.92          | 0.003       | 0.068      | 0.006         | 1.637           | 30.88            | 96.73                      | 63848×2   | 61934×2     | 97                         | 50388      |
| POST3       | 250:250           | 31.62          | 0           | 0.059      | 0.001         | 1.632           | 30.58            | 96.71                      | 63242×2   | 61331×2     | 96.98                      | 53028      |
| PRE1        | 250:250           | 32.25          | 0           | 0.084      | 0.004         | 2.84            | 30.6             | 94.89                      | 64499×2   | 61377×2     | 95.16                      | 60012      |
| PRE2        | 250:250           | 31.48          | 0           | 0.102      | 0.004         | 2.031           | 30.26            | 96.12                      | 62967×2   | 60677×2     | 96.36                      | 59474      |
| PRE3        | 250:250           | 31.52          | 0           | 0.077      | 0.002         | 1.28            | 30.79            | 97.68                      | 63040×2   | 61664×2     | 97.82                      | 47502      |

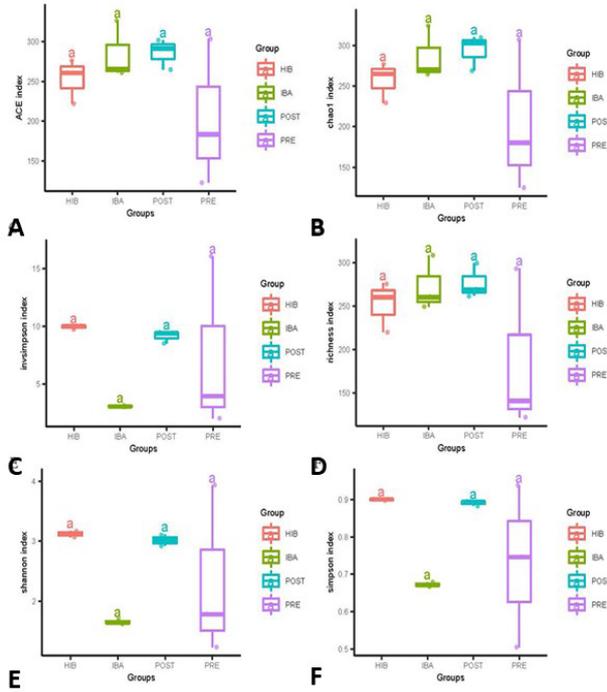


Fig. 2. Venn figure of the OTUs in all groups.

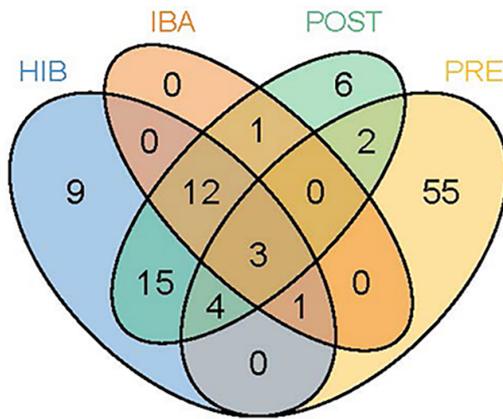


Fig. 3. Comparison of the alpha diversities in all groups. Note: A-F depict the alpha diversity index of ACE, chao1, invsimpson, richness, Shannon, and Simpson, respectively.

The Bray-Curtis distance in each group was calculated. We used unconstrained (principal coordinate analysis, PCoA) and constrained ordinations (constrained PCoA, cPCoA) to show the Bray-Curtis distance in all groups (Fig. 4). The results indicated that HIB and POST groups had very close Bray-Curtis distances whereas in IBA or PRE groups, and the distances were far away. To demonstrate details in the Bray-Curtis distance, we created a heatmap of the Bray-Curtis distance between

each sample (Fig. 5). This clearly depicted the Bray-Curtis distance between each sample. A high similarity was observed in the beta diversities between HIB and POST groups. However, the diversities were different from those in IBA and PRE groups. The Bray-Curtis distance of beta diversity was very close in HIB and POST groups, followed by PRE group.

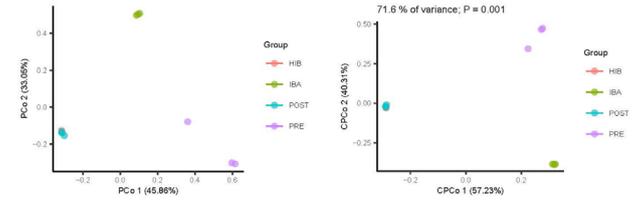


Fig. 4. Beta diversity: Principal coordinate analysis (PCoA) and constrained (PCoA) analyses of Bray-Curtis distance in all groups.

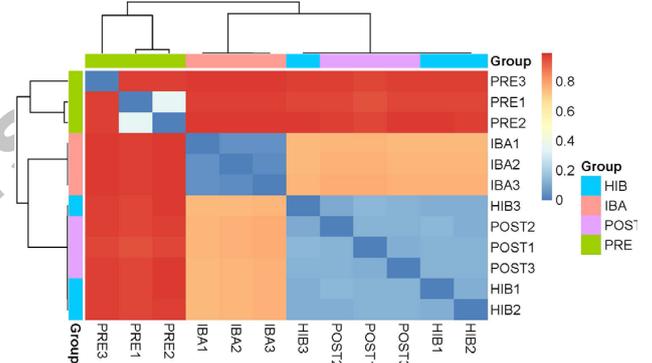


Fig. 5. Beta diversity: Heatmap of the Bray-Curtis distance between each sample.

#### Bacterial community distribution at the phylum level

We counted relative abundance of the Top 7 bacteria in each group at the phylum level (Fig. 6). Before hibernation (PRE group), in *S. dauricus* intestine, Firmicutes was the dominant bacteria, with the highest abundance, followed by verrucomicrobia, bacteroidetes, actinobacteria, and proteobacteria. During the hibernation (HIB group) and post-hibernation (POST group) periods, intestinal bacteria of *S. dauricus* were similarly distributed at the phylum level, and the proteobacteria phylum was the dominant bacteria group, followed by deinococcus-thermus, firmicutes, bacteroidetes, fusobacteria, and others. During the awakening period (IBA group, intestinal ischemic reperfusion period), Firmicutes was the dominant bacterial group followed by proteobacteria, and the relative abundances of bacteroidetes, deinococcus-thermus, verrucomicrobia, and other phyla were very low in the

intestine of *S. dauricus*. It was observed that the phylum intestinal bacterial composition of *S. dauricus* significantly changed during the hibernation period compared with the pre-hibernation period. The bacterial abundance of proteobacteria and deinococcus-thermus phylum increased while that of firmicutes and Verrucomicrobia decreased. In the awakening period (IBA group), the composition of the bacterial community changed more drastically and the relative abundance of Firmicutes and Proteobacteria increased sharply while that of other bacteria decreased. Additionally, in the POST group (2 days after the end of hibernation), the composition of the intestinal bacterial phyla of *S. dauricus* was similar to that in the hibernation period, and it did not recover in the pre-hibernation period. It might be expected that it would take some time for the bacterial composition to adjust and recover.

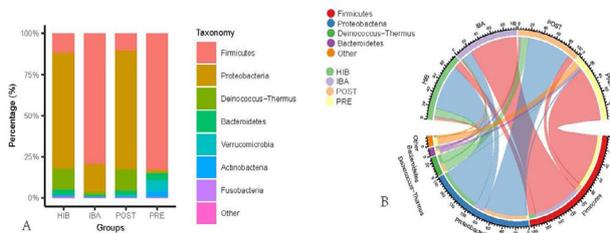


Fig. 6. Bacterial community distribution at the phylum level. A, Stacked histogram of the intestinal bacterial abundance in each group at the phylum level (top 7). B, Chord diagram of intestinal bacterial abundance in each group at the phylum level (top 4).

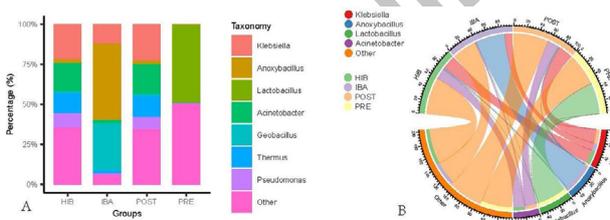


Fig. 7. Bacterial community distribution at the genus level. A, Stacked histogram of intestinal bacterial abundance in each group at the genus level (top 7). B, Chord diagram of intestinal bacterial abundance in each group at the genus level (top 4).

#### Bacterial community distribution at the genus level

Similarly, we counted the relative abundance of the Top 7 bacteria in each group at the genus level to explore the changes (Fig. 7). In the PRE group, *Lactobacillus* was the dominant genus with the highest intestinal abundance. The HIB group also had a similar bacterial construction at the genus level as that in the POST group; *Klebsiella*

was the predominant bacteria, followed by *Acinetobacter*, *Thermus*, and *Pseudomonas*. However, in the IBA group, *Anoxybacillus* was the dominant genus, followed by *Geobacillus* and *Klebsiella*, and the relative abundances of other bacteria were low. Compared to the pre-hibernation period, intestinal bacteria at the genus level significantly changed during the hibernation period. The abundance of *Lactobacillus* decreased sharply while the abundance of *Klebsiella*, *Acinetobacter*, *Thermus*, *Pseudomonas*, and *Anoxybacillus* increased sharply. Particularly, during the awakening period (IBA group), the abundance of *Lactobacillus* decreased sharply, and the abundance of *Anoxybacillus* and *Geobacillus* increased sharply, making them the main bacteria. Similarly, in the POST group, the composition of intestinal bacteria was still similar to that in the hibernation period.

#### The main OTU evolutionary tree of intestinal bacteria

We constructed a phylogenetic tree for the top 100 OTUs with relative abundances above 0.2% and performed relative annotations. The results were shown in Figure 8. It showed the expression of the top 100 OTUs in each group and/or sample with the class phylum to family. Among the top 100 OTUs, Firmicutes had the highest abundance, followed by Proteobacteria, and the rest were Bacteroidetes and Actinobacteria.

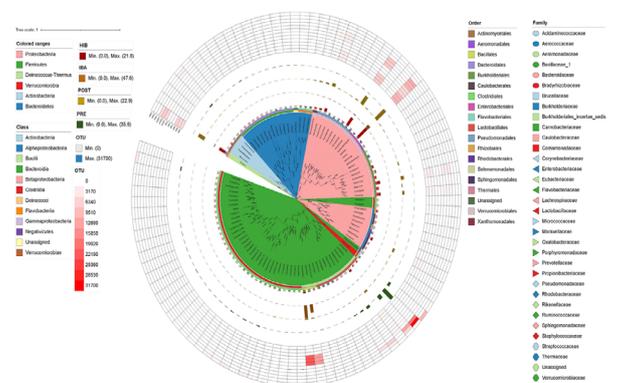


Fig. 8. The main OTU evolutionary tree of intestinal bacteria. The OTU evolutionary tree was constructed using the Top 100 OTUs with a relative abundance greater than 0.2%. The branches of the evolutionary tree were filled with color using phylum-level bacterial classification. OTUs used the genus level to correspond to bacterial annotations. The circles of evolutionary tree from inside to outside with color bands or blocks were at the level of class, order, and family. The next level was a bar graph representing the expression level of OTUs in HIB, POST, and PRE groups. The outermost layer was a heat map of the corresponding OTU abundance in each sample.

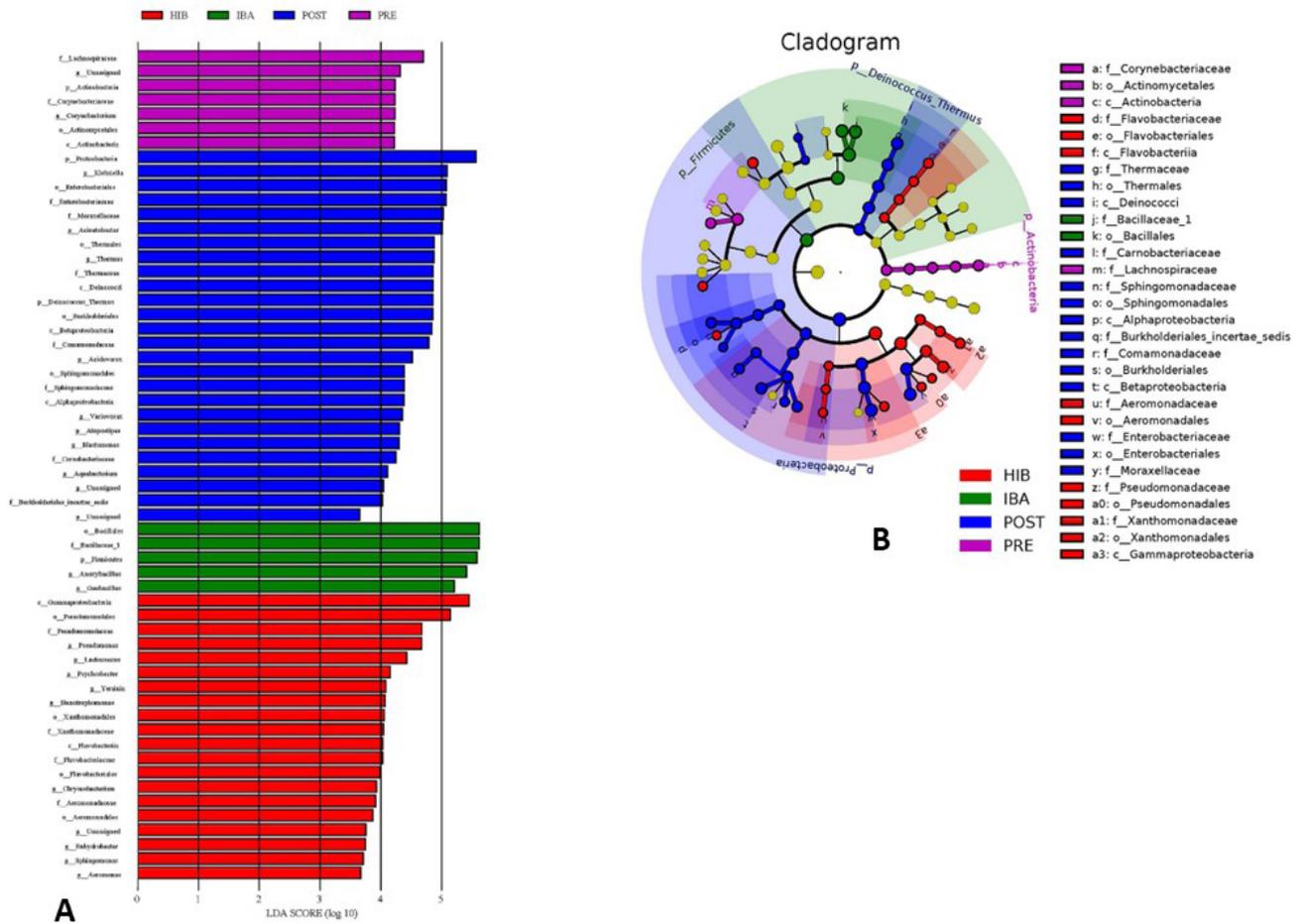


Fig. 9. Biomarkers predicted in LefSe. A, LefSe result in bar; B, LefSe result in Cladogram.

### LefSe analysis for biomarkers

To predict the possible differential biomarker bacteria of the intestinal bacteria of *S. dauricus* in different hibernation periods, LefSe analysis was conducted, and the results are shown in Figure 9. HIB and POST groups had 26 (*p*\_Proteobacteria, *g*\_Klebsiella, *o*\_Enterobacteriales, *etc.*) and 20 (*c*\_Gammaproteobacteria, *o*\_Pseudomonadales, *f*\_Pseudomonadaceae, *etc.*) differential biomarkers, respectively. PRE group had seven biomarkers, namely *f*\_Lachnospiraceae, *p*\_Actinobacteria, *f*\_Corynebacteriaceae, *g*\_Corynebacterium, *o*\_Actinomycetales, *c*\_Actinobacteria, and one *g*\_unassigned. The IBA group contained only four biomarkers, *o*\_Bacillales, *f*\_Bacillaceae\_1, *p*\_Firmicutes, *g*\_Anoxybacillus, and *g*\_Geobacillus.

### Comparison of the main differences in OTU and bacterial abundance among IBA group and PRE and HIB groups

Previous beta diversity results had suggested that the bacterial compositions of HIB and POST groups were

relatively similar. Therefore, the HIB group was used for comparison with other groups. OTUs play a key role in IBA, PRE, and HIB groups, the significant OTU abundance and its corresponding bacteria were analyzed between multiple groups. As shown in Figure 10A, compared to the bacteria in PRE group, the bacteria in IBA group had a higher abundance of 16 OTUs, such as OTU7, OTU49, and OTU27, and lower abundance of 12 OTUs, including OTU45, OTU54, and OTU18. Similarly, as shown in Figure 10B, compared to the PRE group, the bacteria in IBA group had a lower abundance of 20 OTUs such as OTU10, OTU11, and OTU2 and higher abundance of four OTUs, such as OTU3, OTU4, OTU1, OTU55, and OTU31.

After annotating these OTUs as the corresponding bacteria, we compared the abundance of the main bacteria in IBA group with that in PRE and HIB groups at the phylum (Fig. 11) and genus levels (Fig. 12). We counted the bacteria with significant differences in abundance.

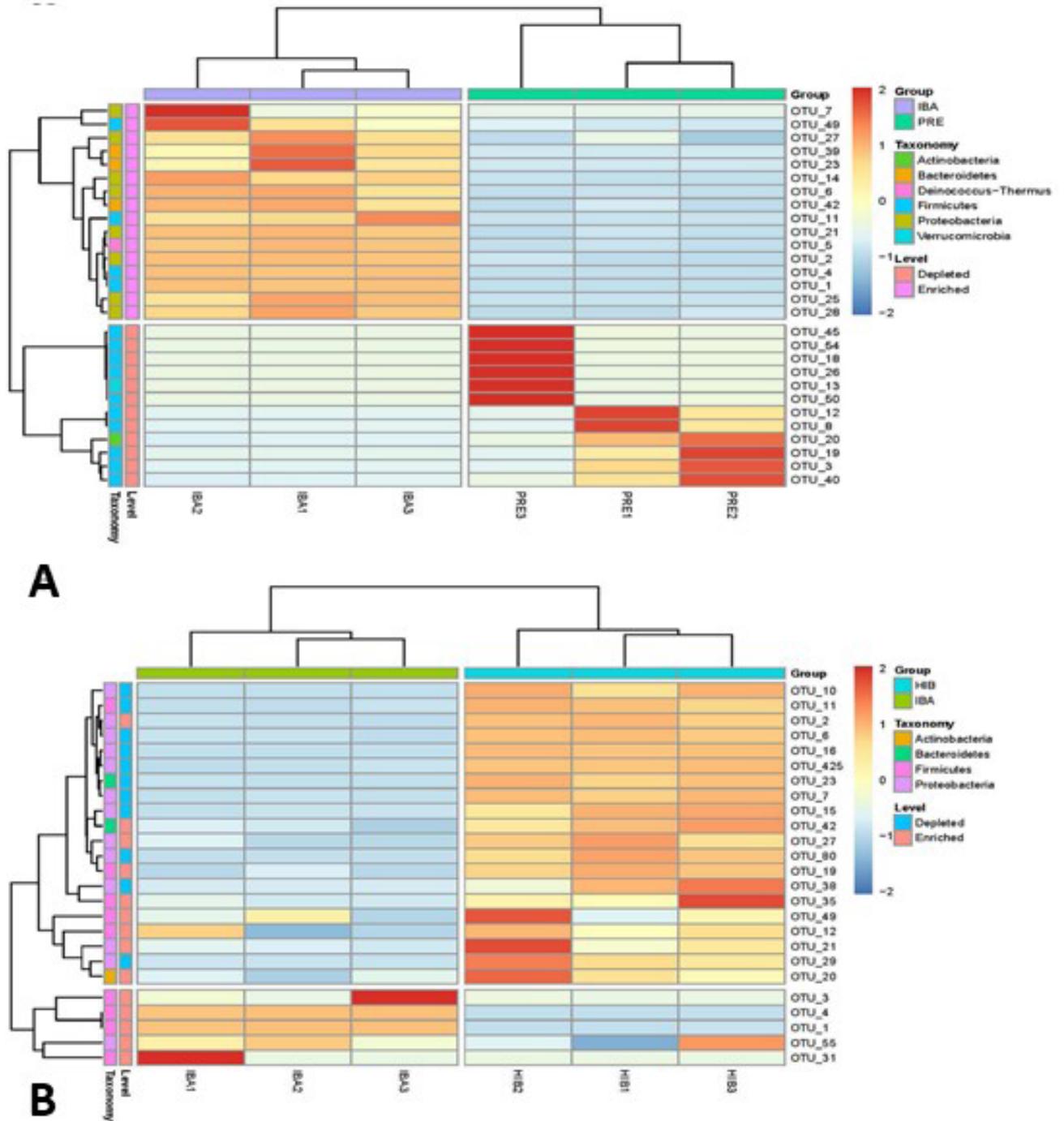


Fig. 10. Heatmap of the OTUs with significantly different abundances in IBA, PRE, and HIB groups. A, Significant difference in the OTU abundance between IBA and PRE groups. B, Significant difference in the OTU abundance between IBA and HIB groups.

At the phylum level (Fig. 13), the bacterial abundance of *Actinobacteria* in PRE group was significantly higher than that in HIB, IBA, and POST groups ( $P < 0.05$ ). The bacte-

rial abundance of *Deinococcus-Thermus* in POST group was significantly higher than that in PRE and IBA groups ( $P < 0.05$ ), and its bacterial abundance was significantly

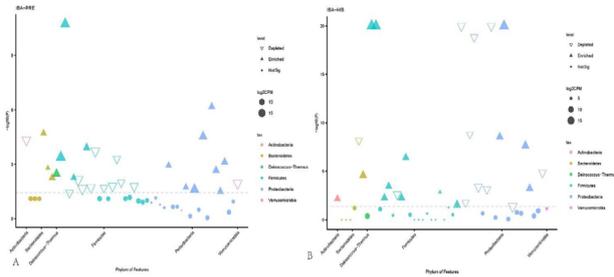


Fig. 11. Manhattan chart of significant bacterial abundance at the phylum level in IBA, PRE, and HIB groups.

higher in HIB than that in PRE and IBA groups ( $P < 0.05$ ). The abundance of *Firmicutes* bacteria in POST group was significantly lower than that in PRE and IBA groups ( $P < 0.05$ ); however, it was significantly lower in HIB group as compared to that in PRE and IBA groups ( $P < 0.05$ ). The abundance of Proteobacteria was significantly lower in PRE group than that in POST and IBA groups ( $P < 0.05$ ); however, its abundance was significantly higher in POST group than that in IBA group ( $P < 0.05$ ). Moreover, its abundance was significantly higher in HIB group than that in PRE and IBA groups ( $P < 0.05$ ).

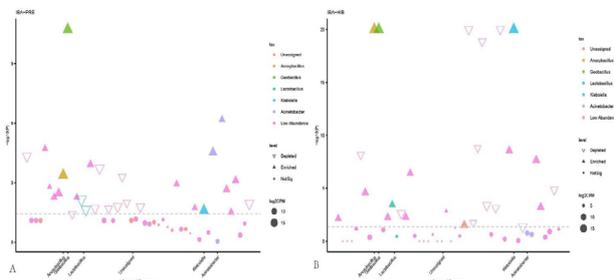


Fig. 12. Manhattan chart of significant bacterial abundance at the genus level in IBA, PRE, and HIB groups.

At the genus level (Fig. 14), compared to the abundance in PRE group, the abundance of 11 kinds of bacteria, including *Anoxybacillus*, *Geobacillus*, *Klebsiella*, *Thermus*, and *Acinetobacter*, in IBA group significantly increased ( $P < 0.05$ ). Meanwhile, compared to the abundance in HIB group, the abundance of *Anoxybacillus* and *Geobacillus* in IBA group significantly increased ( $P < 0.05$ ). However, the abundance of 16 species of bacteria, including *Acinetobacter*, *Aquabacterium*, *Acidovorax*, *Thermus*, and *Klebsiella*, significantly decreased ( $P < 0.05$ ). Compared to the abundance in PRE group, the abundance of *Klebsiella*, *Pseudomonas*, *Acinetobacter*, *Thermus*, and *Acidovorax* in HIB group significantly increased ( $P < 0.05$ ).

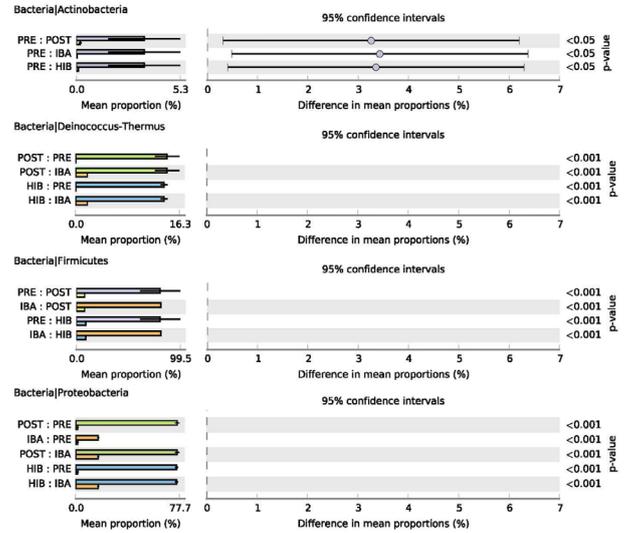


Fig. 13. Significant differences in bacterial abundance at the phylum level.

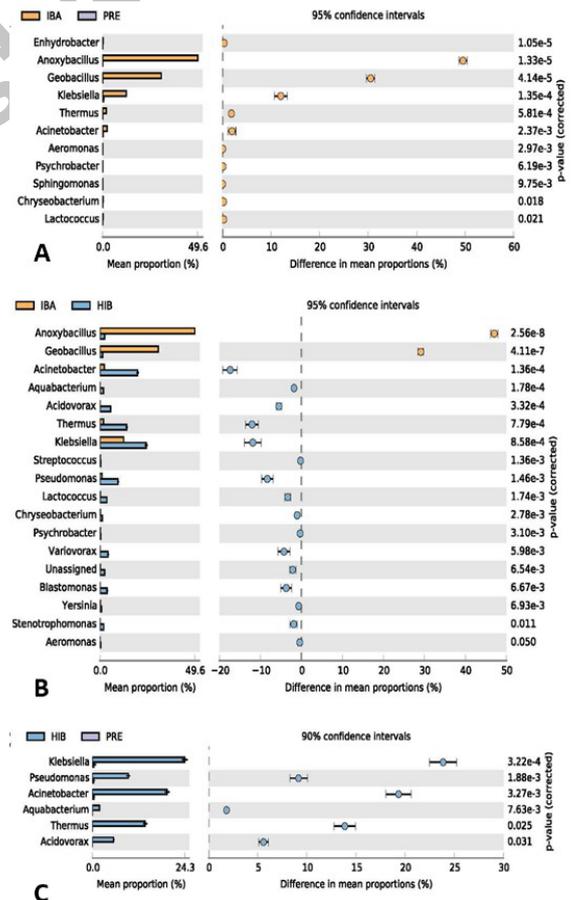


Fig. 14. Significant differences in bacterial abundance at the genus level.

## DISCUSSION

In this study, we found that fewer OTUs were shared between the pre-hibernation and other periods of hibernation as compared to common OTUs in each period of hibernation. Additionally, although the beta diversity distance of *S. dauricus* gut microbes was obvious, no significant difference was observed in the alpha diversity at each period. The reason for this remains unclear and further research is required.

Hibernation is an adaptation of hibernators. It can alter the intestinal epithelial, immune, and cell survival pathways (Kurtz *et al.*, 2021). Under hibernation fasting and cold conditions, the intestinal microbial community adaptively reorganizes to maintain physiological functions, and the intestinal microbiota abundance, composition, and diversity may change. Therefore, studies have analyzed changes in the intestinal microbial community of hibernating animals during hibernation to provide a new perspective for the treatment of intestinal diseases, including ischemia/reperfusion injury (Sisa *et al.*, 2017). Since no food is supplied during hibernation, it is foreseeable that the gut microbial community of hibernating animals will change during different hibernation periods. In this study, prior to hibernation, Firmicutes was the dominant bacteria in the gut of *S. dauricus*, and its abundance decreased during hibernation; however, Bacteroidetes showed an opposite trend. The results were similar to those of the bacterial communities in hibernating ground squirrels and brown bears (Dill-McFarland *et al.*, 2015; Carey *et al.*, 2013; Sommer *et al.*, 2016). Compared to before hibernation, during post hibernation, the bacterial community composition of *Bombus terrestris* intestine was higher and abundance of the core bacteria *Gilliamella* (Proteobacteria, *Orbaceae*) and *Snodgrassella* (Proteobacteria, *Neisseriaceae*) decreased but that of other non-core bacteria increased (Bosmans *et al.*, 2018). Similarly, in this study, *Geobacillus* was also the core bacteria, especially the second most dominant community in the awakening period in IBA group. However, compared to the bacteria before and during hibernation, the *Geobacillus* of *S. dauricus* intestine increased during the awakening period. These changes might cause hibernation to affect lipid metabolism, such as triglyceride, cholesterol, and bile acid metabolism (Sommer *et al.*, 2016). Further, it may convert the metabolic function of intestinal microbes from carbohydrate-related to lipid-related functional categories (Xiao *et al.*, 2019) and change the microbial short-chain fatty acids and other metabolite features (Carey and Assadi, 2017).

The hibernators had core OTUs that were rarely

distributed among non-hibernating animals, and hibernation might reduce the microbial diversity in frogs (Weng *et al.*, 2016).

## CONCLUSIONS

This study demonstrated changes in the diversity of intestinal microbial communities before, during, and after the hibernation and awakening periods. The results showed that there was no significant change in alpha diversity during the four periods; however, the beta diversity was different, especially in the awakening period. The differences in beta diversity may be due to the fact that hibernation can increase the abundance of four species of bacteria, such as *Klebsiella*, in the intestine of *Spermophilus dauricus*, as compared to before hibernation. The arousal (ischemic reperfusion) phase may lead to an increase in bacterial abundance in the intestine, such as that of *Anoxybacillus*. Compared to hibernation, the awakening (ischemic reperfusion) phase leads to an increase in the abundance of *Anoxybacillus* and *Geobacillus* and a decrease in the abundance of 16 species of bacteria, including *Acinetobacter*, in the intestines of *S. dauricus*. Although we know that the intestinal bacterial community is closely related to animal behavior and physiological metabolism, this study also found that the intestinal community significantly changes during hibernation; however, the specific interaction mechanism is still unclear. Nevertheless, this study provides guidance and reference for the exploration of animal hibernation mechanisms and research on intestinal ischemic re-entery injury.

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### Institutional review board statement

Ethical review and approval were waived for this study, due collection samples from intestinal feces, which was a non-invasion method.

### Data availability statement

Not applicable.

### Statement of conflict of interest

The authors have declared no conflict of interest.

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