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Microbial-feeding interactions reveal the effects of feeding blood on the gut microbiota of the aquaculture leech (*Hirudo nipponica)*

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Abstract

Leeches (*Hirudo nipponica*), as a kind of aquatic animal, mainly feed on fresh blood. After feeding, they needed to digest for a long time because the intestinal digestive enzyme content is low, so their digestive needed the help of gut microbiota. Here, we examined intestinal microbiota in captive *Hirudo nipponica* of different periods after feeding blood with high-throughput sequencing. The results showed that gut microbial diversity was lower before feeding than after. At the level of the core phylum of the gut microbiota of *Hirudo nipponica*, the focus was on Proteobacteria, Bacteroidetes, and Firmicutes. After feeding blood, the relative abundance of Proteobacteria decreased, while the opposite was true for Bacteroidetes and Firmicutes. The core bacteria at the genus level are Aeromonas and Mucinivorans. The results show that the structure of the gut microbiota and function are closely associated with the blood feeding. The study aimed to lay a theoretical foundation for the blood-digestive mechanism of *Hirudo nipponica*.

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Abbreviations

H. nipponica: *Hirudo nipponica*
H. verbana: *Hirudo verbana*
M. hirudinis: *Mucinivorans hirudinis*
H. medicinalis:*Hirudo medicinalis*
M. decora:Macrobdella decora,
HPC0: the un-ingested group
HPC1: the 1 d after blood feeding group
HPC3: the 3 d after blood feeding group
HPC5: the 5 d after blood feeding group
HPC7: the 7 d after blood feeding group
HPC10: the 10 d after blood feeding group
HPC20: the 20 d after blood feeding group
HPC30: the 30 d after blood feeding group

Introduction

Many microbial communities exist in the animal gut while forming a complex ecosystem in the host and playing important roles in nutrient digestion and absorption (Upadhyay et al., 2012), regulation of host immune function (Kaltenpoth et al., 2014), and defense against harmful microbial attack (Hu et al., 2021). The gut microbiota, a hot spot of aquatic animal research (Miao et al., 2020), can help the host digest food and synthesize nutrients that the animal host cannot synthesize. For instance, *Bacillus velezensis* in the gut of *Procambarus clarkii* can secrete cellulase and promote its digestion of herbivorous feed (Feng et al., 2020). *Sphingomonas*, the dominant flora in the gut of *Brachymystax lenok*, can promote digestion (Huang et al., 2018). These studies demonstrate that gut microbiota plays a crucial role in digestion.

To date, structural changes in the gut microbiota of aquatic animals after feeding are still poorly understood. Traditional gut microbiota research methods are mainly in vitro culture methods, but this method has a downside. Since most intestinal microorganisms are difficult to culture in vitro, they cannot be followed up with further studies. High-throughput sequencing bridges this gap, allowing for sequencing analysis and strain identification of highly variable regions. Moreover, this technique provides a more comprehensive understanding of the composition of microorganisms in the gut and their abundance. It facilitates the study of microorganisms that are not easily cultured in the gut (Wang et al., 2015). Research shows that the gut microbiota of aquatic animals such as *Eriocheir sinensis* (Wang et al., 2019) and *Ctenopharyngodon idellus* (Tran et al.,2018) have been studied and analyzed by high-throughput sequencing under two states, starvation and prefeeding. It was found that feeding affects gut microbiota's diversity and structural composition.

Hirudo nipponica Whitman (*Hirudo*, Hirudinidae, Arhynchobdellida) is an invertebrate confined to the Asian continent that is widely distributed in China and is listed as an animal raw material for Chinese medicinal materials. The use of leeches in medicine has been documented, and they have medicinal value, such as anticoagulation and thrombolytic (Baskova et al., 2001). *H. nipponica* mainly feeds on the blood of mammals, and the amount of food consumed is notable, more than five times their body weight at a time. Moreover, the nutrients in the blood are the material basis for its growth and development. According to Sawyer (1986), the digestion and absorption of blood in the digestive tract of leeches after ingestion relies mainly on intestinal symbiotic bacteria, as the leeches lack heme-degrading proteases, thus providing nutrients for their growth and development. This indicates that the gut microbes of leeches are important for digestion and vital for their growth and development.

Therefore, high-throughput sequencing of the gut microbes of leeches has been performed in recent years. To date, the gut microbiota of *Hirudo orientalis* (Whitaker et al., 2014), *Hirudo medicinalis* (Graf et al., 1999), *Hirudo verbana* (Nelson et al., 2012), and *Macrobdella decora* (Siddall et al., 2007) have been analyzed and reported. Studies on the gut microbiota of *H. nipponica* are rare, and most of them are on intestinal symbionts during starvation and refeeding periods (Li et al., 2021; Shi et al., 2019), but few systematic studies have been conducted on the gut microbiota of Japanese medical leeches after feeding. Therefore, our groups investigated the structural changes in the gut microbiota of *H. nipponica* after blood feeding and compared them with the post-feeding samples using high-throughput sequencing technology, intending to lay a theoretical foundation for an in-depth study of the gut microbiota and digestive mechanism of the leeches.

Materials and Methods

Animal ethics statement

All handling of *Hirudo nipponica* was conducted following the Guidelines for the Care and Use of Animals for Scientific Purposes recommended by the Institutional Animal Care and Use Committee (IACUC) of Yancheng Institute of Technology, China.

Sample collection

Animals were collected in Dalian, Liaoning Province, China. Healthy, uninjured, similarly sized leeches were selected as experimental animals with an initial weight of 0.30±0.15 g. All experimental animals were starved for 30 d before feeding, and there were eight groups in the experiment. The control group was only treated with starvation. The test groups were fed leeches after starvation treatment, and the feed was fresh, healthy porcine blood, which was injected into fresh, healthy pig intestines for feeding. In the control group, the 20 *H. nipponia* intestines and intestinal contents were prepared as a mixed sample (HPC0). After feeding blood, *H. nipponia* was anesthetized on ice in a sterile environment, and the body surface was wiped with 75% alcohol for disinfection and dissection. The intestines and the contents at 1 d, 3 d, 5 d, 7 d, 7 d, 10 d, 20 d, and 30 d were collected after feeding and recorded as HPC1, HPC3, HPC5, HPC7, HPC10, HPC20, and HPC30, respectively, and 20 leeches were repeatedly selected from each group for mixing. The gut samples taken from each leech were 0.05g. The water temperature was maintained at 21-25°C throughout the breeding period.

DNA extraction amplicon generation and high-throughput sequencing

The intestinal tissue samples were pulverized, and 0.5 g of the sample powder was placed in a 1.5 mL microcentrifuge tube. Total genomic DNA from samples was extracted using the SDS method. DNA concentration and purity were monitored on 1% agarose gel. Universal bacterial barcoded primers (515F: 5′-GTGCCAGCMGCCGCGGTAA-3′, 806R: 5′-GGACTACVSGGGTATCTAAT-3′) and a thermocycler system (GeneAmp 9700, ABI, Foster City, CA, USA) were used to perform polymerase chain reaction (PCR) amplification of V4 hypervariable regions of the 16 S rRNA gene, and the reactions were performed in triplicates. All PCRs were carried out with 15 μL of Phusion® High-Fidelity PCR Master Mix (New England Biolabs), 0.2 μM forward and reverse primers, and approximately 10 ng of 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, and elongation at 72°C for 30 s. Finally, the PCR product was used for high-throughput sequencing. High-throughput sequencing was performed on 16S rRNA V4 region with MiSeq (Illumina, San Diego, CA, USA).

Data analysis and availability

The paired-end reads were merged after truncation using FLASH (2011) (Magoč et al., 2011), and the splicing sequences were called raw tags. Quality filtering of the raw tags was performed to obtain high-quality clean tags according to QIIME software (version 1.9.1) (Caporaso et al., 2010) quality control. The tags were compared with the Silva database (Quast, et al., 2013) using the UCHIME algorithm (Edgar, et al., 2011) to detect chimera sequences and finally obtain the effective tags. The effective tags were subjected to primer sequence removal and clustering for the generation of operational taxonomic units (OTUs) with Uparse software (version 7.0.1001) (Edgar 2013) with >97% similarity. For each representative sequence, the Silva Database (Quast 2013) was used based on the Mothur algorithm to annotate taxonomic information (confidence threshold was 0.8-1.0). Using MUSCLE (Version 3.8.31) software for multiple sequence alignment, get all OTUs on behalf of the sequence of the system. The phyloseq package of R software was used to import data and calculate the richness index of Chao1 and the diversity indices of Simpson's and Shannon based on the OTU table. Use MUSCLE (Version 3.8.31) for multiple sequence alignment, get all OTUs representative sequence phylogenetic relationship of the evolutionary tree. Principal

component analysis (PCA) and nonmetric multidimensional scaling (NMDS) were used to compare microbial construction differences between different feeding samples.

Function prediction

Extract the 16S rRNA genome sequence of prokaryotes in the KEGG database and compare it using the BLASTN algorithm to the SILVA SSU Ref NR database (BLAST bitscore > 1500) to establish a correlation matrix. The genome-wide functional information of prokaryotes from the KEGG database annotated by both the UProC and PAUDA methods was obtained corresponding to the SILVA database and functionally annotated.

Results

OTUs and feasibility analysis

A total of 663,738 original sequences were obtained from the 8 samples, and 594,107 valid sequences were filtered, which could be clustered into 638 classification operation units (OTUs). The number of common OTUs in all samples was 90, the number of HPC0-specific OTUs was 308, the number of HPC1-specific OTUs was 55, the number of HPC10-specific OTUs was 22, the number of HPC0-specific OTUs was 16 (**Figure 1**), and the overall trend was downward with increasing time.

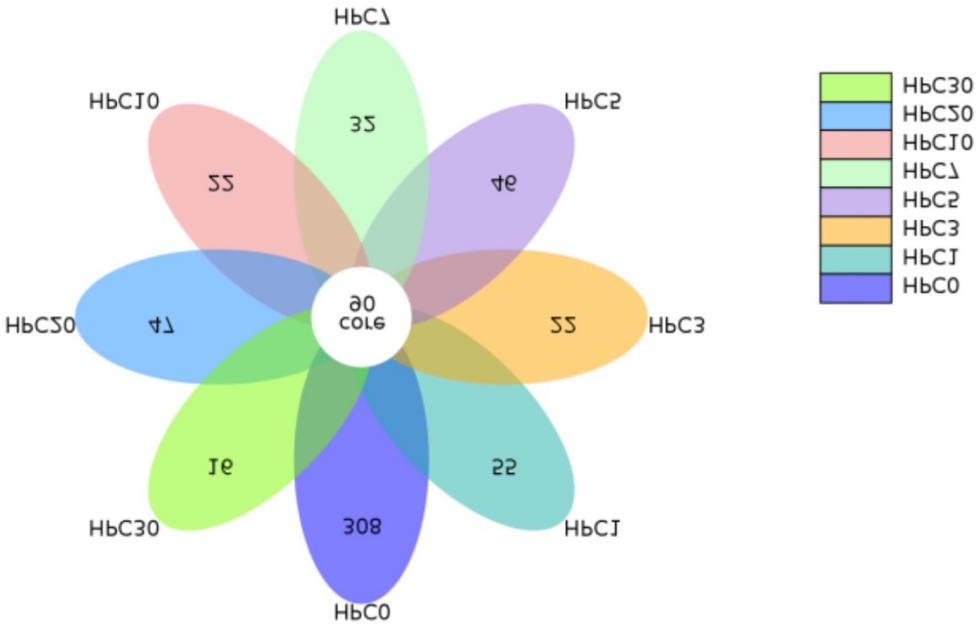

Figure 1 OTUs Venn diagram of gut microbiota of *Hirudo nipponica* at different feeding periods.

The rarefaction curves (**Figure 2A**) showed that the curves plateaued across all samples, indicating that the test sequencing depth included the microbial diversity of all samples. The rank abundance curves (**Figure 2B**) showed that the curves had more span on the horizontal axis and less smoothness on the longitudinal axis, indicating relatively high species richness and uniform distribution in the samples.

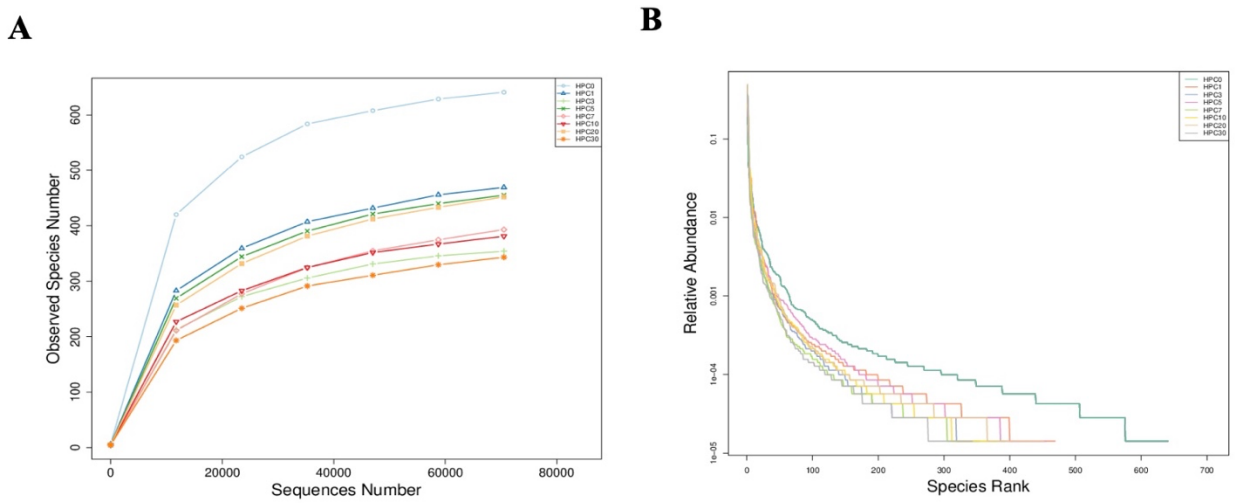

Figure 2 The Rarefaction curves (A) and the rank clustering curves (B) for different feeding periods.

Microbial community diversity

The α diversity analysis index showed that all samples had 99.9% coverage. The Shannon indices (**Figure 3A**), ACE indices (**Figure 3C**), and Chao1 indices (**Figure 3D**) in the HPC0 group were higher than those in the remaining seven groups after feeding blood, the Simpson indices after 3 days of feeding showed a downward trend, and the value was lower than HPC0 (**Figure 3B**).

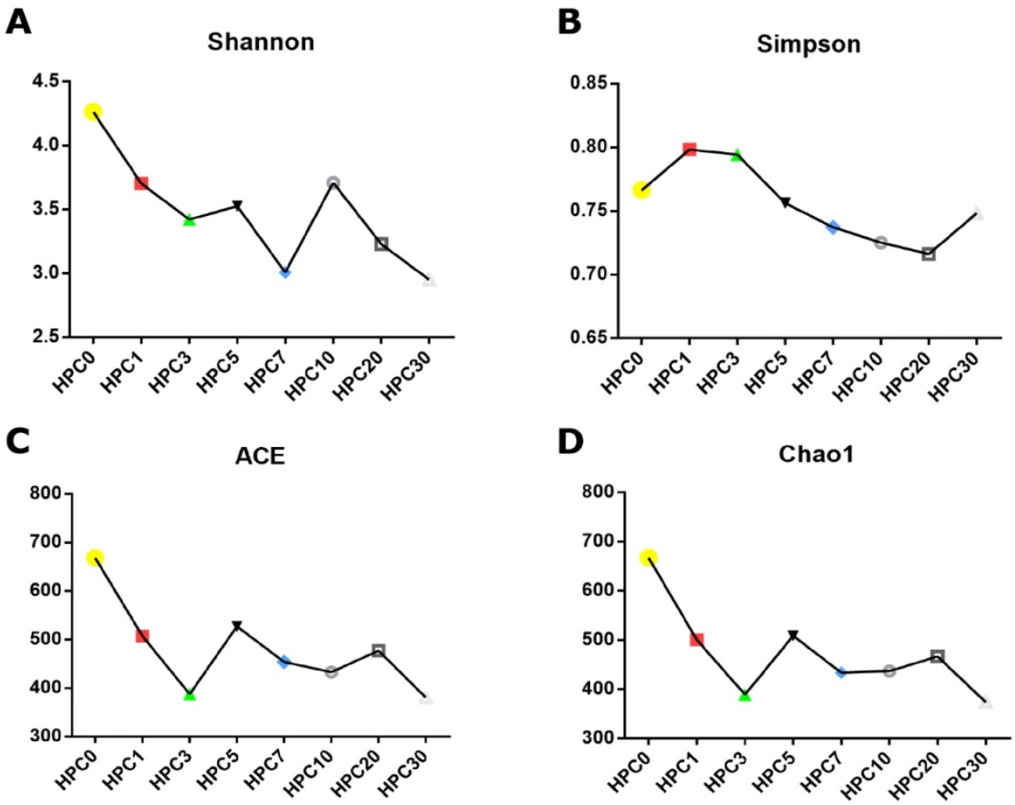

Figure 3 The α diversity indices for different feeding periods. Note: The Shannon indices (A), the Simpson indices (B), the ACE indices (C), and the Chao1 indices (D).

PCA of different feeding periods based on OTU levels (**Figure 4A**). The HPC1, HPC3, HPC5, HPC7, HPC10, HPC20, and HPC30 groups were located near each other in the PCA picture, but all were far from HPC0. According to NMDS statistical analysis (multidimensional calibration) (**Figure 4B**), the HPC1, HPC3, and HPC5 groups, as well as an additional post-feeding blood group, overlapped in the NMDS map, and all were far from the HPC0 samples, indicating that the difference between the intestinal flora structure of *H. nipponia* before and after feeding blood was great.

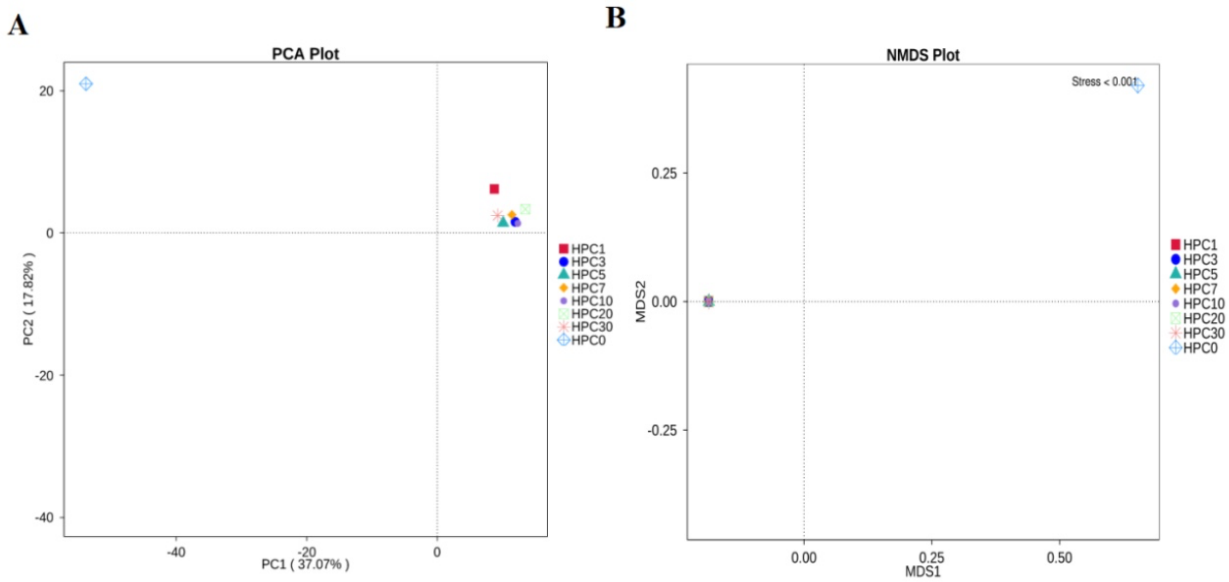

Figure 4 PCA analysis (A) and NMDS analysis (B) based on OTU levels during different feeding periods.

Composition of the gut microbiota communities at different levels

At the phylum level (**Figure 5A**), the gut microbiota structure of *H. nipponia* before and after ingestion of blood meal was generally similar. All samples had three dominant phyla: Bacteroidota, Proteobacteria, and Firmicutes. The relative abundance of these three dominant phyla together accounted for more than 97% of the total phyla, and the percentage of other phyla was only 3%. In the pre-feeding blood gut microbiota, Proteobacteria was the most dominant phylum, with a relative abundance of 61.55%. Following Bacteroidota, the relative abundance was 18.40%. The next most abundant phylum was Firmicutes, with a relative abundance of 6.75%. The relative abundance of Proteobacteria in the gut microbiota decreased after blood meal feeding, decreasing to a minimum after 30 d, and the relative abundance was 24.37%. Bacteroidota and Firmicutes' relative abundances were higher than before blood feeding. The relative abundance of Bacteroidota increased to the highest after 20 d, reaching 57.45%. After 30 d of blood meal feeding, the relative abundance of Bacteroidota decreased to 33.67%. Moreover, the relative abundance of Firmicutes peaked after 30 d (40.42%). Of the 10 genera with the highest relative abundances in the *H. nipponia* gut microbiota (**Figure 5B**), the genus with the highest relative abundance before blood meal feeding was *Ralstonia*, with a relative abundance of 47.62%. The second-most abundant genus was *Prevotella_9,* with a relative abundance of 6.54%. The third-most abundant genus was *Bacteroides,* with a relative abundance of 4.99%. In the other post-feeding blood groups, the relatively high-abundance genera were *Mucinivorans* and *Aeromonas*, with average abundances of 32.15% and 20%, respectively. The evolutionary relationship also shows that there are mainly *Mucinivorans* and *Aeromonas* in the gut after ingestion compared to before feeding (**Figure 5C**).

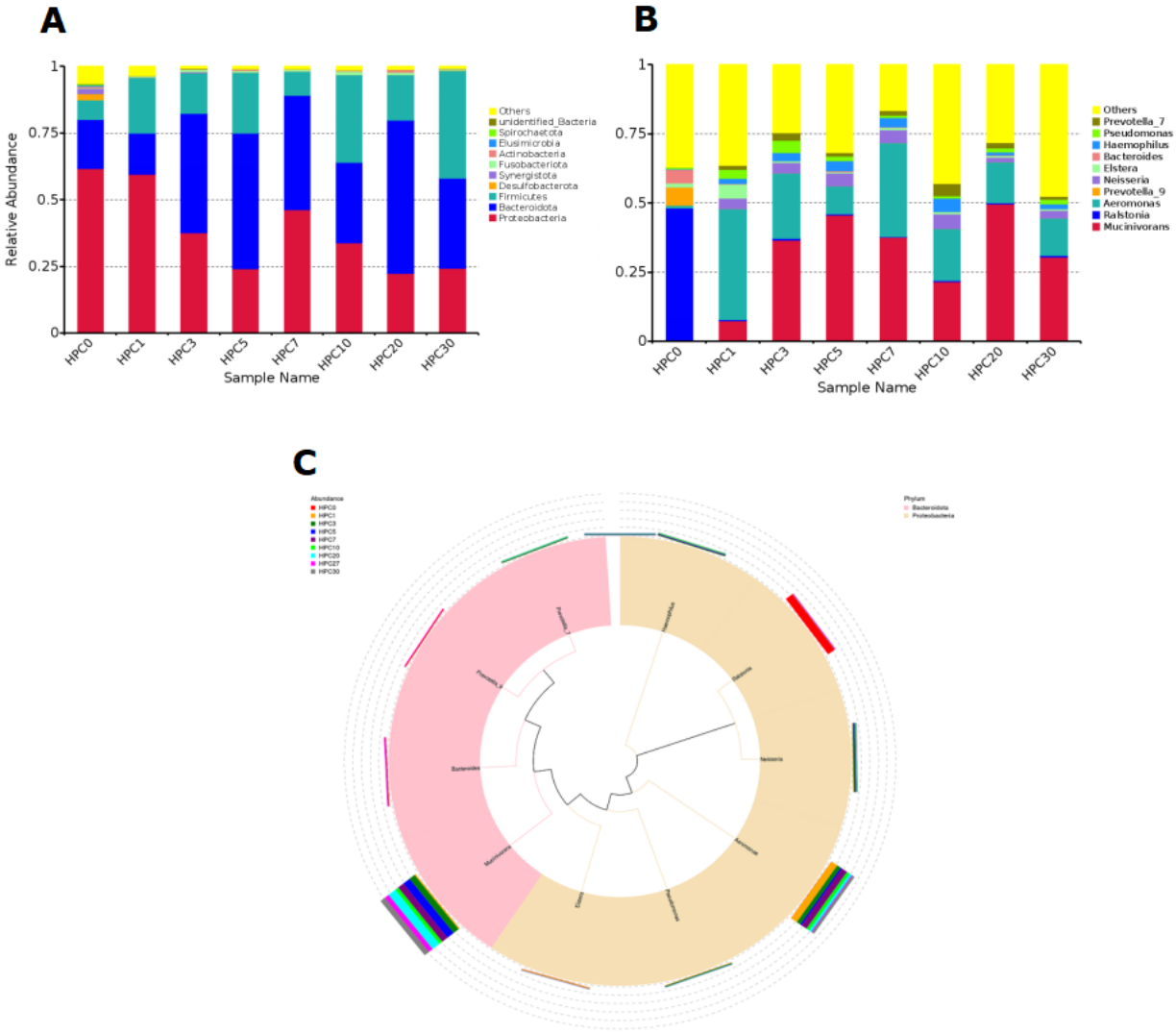

Figure 5 Histogram of top 10 relative abundance at the phylum level (A), histogram of top 10 relative abundance at the genus level (B), phylogenetic trees constructed from representative sequences at the genus level (C) in different feeding periods.

Function prediction

Tax4Fun function prediction was performed on 8 samples by the nearest neighbor method based on minimum 16S rRNA sequence similarity. The changes in the level 1 KEGG metabolic pathway showed that compared with before blood meal feeding (**Figure 6A**), the metabolism (44.49%), cellular process (7.76%), human disease (3.22%), and a biological system (1.69%) in the seven groups, but the abundance of genetic information processing (21.79%) and environmental information processing (15.37%) pathways increased significantly. The level 2 KEGG metabolic pathway showed an increased abundance of carbohydrate metabolism (**Figure 6B**), endocrine system, endocrine and metabolic disease, energy metabolism, nucleotide metabolism, lipid metabolism, metabolism of cofactor and vitamin, xenobiotic biodegradation and metabolism, signal transduction, cellular processes and signaling, and aging pathways

after blood meal feeding.

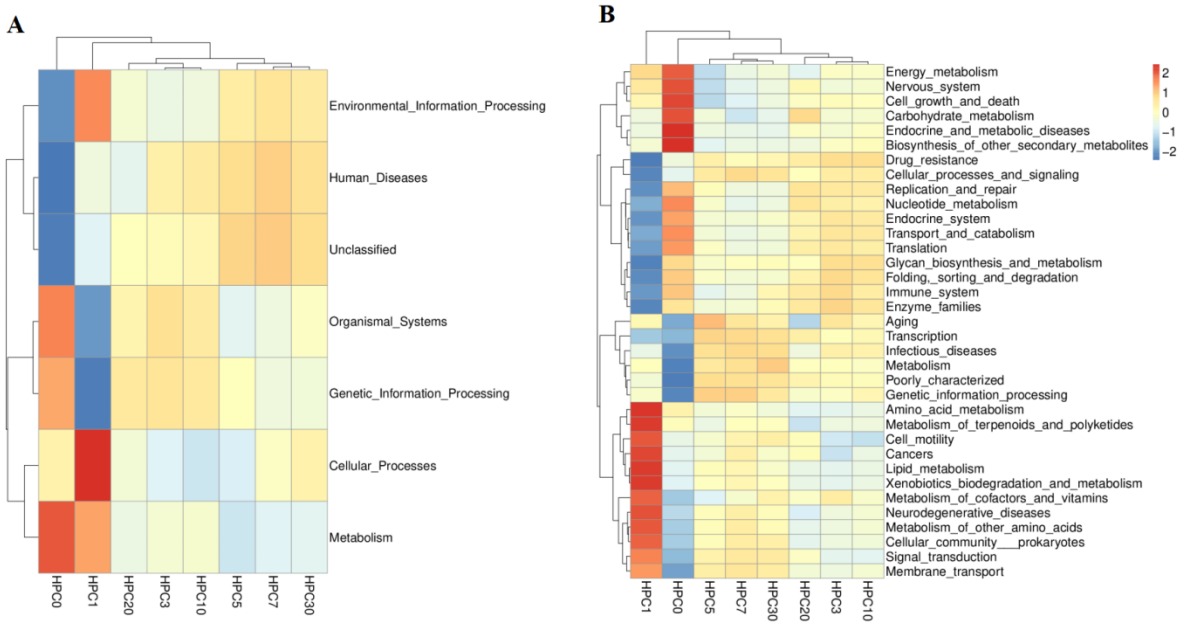

Figure 6 Predicted functional gut microbial community at different feeding periods. Note: KEGG primary metabolic pathway predicts functional gene clustering heat map (A), KEGG secondary metabolic pathway predicts functional gene clustering heat map (B).

Discussion

The species and quantity of the gut microbiota will change with the time of breeding, food composition, and breeding environment (Kong et al., 2019). PCA and NMDS analysis showed low similarity between the gut microbial structure of *H. nipponia* before and after 30 d of ingestion, indicating that blood ingestion had a greater effect on the gut microbial structure of *H. nipponia* than 30 d after feeding. With increasing time after feeding blood, the microbial diversity in the gut of *H. nipponia* was reduced, and this trend was like the trend in the gut microbial diversity in post-starvation feeding experiments conducted in *Eriocheir sinensis* (Wang et al., 2015) and *Ctenopharyngodon idellus* (Shen et al., 2019; Tran et al., 2018). In addition, feeding was also shown to increase competition between gut microbial species, accelerating the formation of dominant species and leading to a differentiation in the species and abundance of each microorganism, with beneficial effects on the host. For example, in *Elopichthys bambusa*, after feeding, *Bacillus subtilis* was the dominant bacterium in its gut (Zhong et al., 2016), which was able to enhance host intestinal digestive enzyme activity and improve the immunity of the organism (Shen et al., 2013). It is therefore hypothesized that the decrease in the gut microbial diversity after feeding by *H. nipponia* may also be related to the formation of dominant microbial populations in the gut.

In the present study, the dominant bacterial phyla in the gut of *H. nipponia* at both the pre-feeding blood and post-feeding blood stages were Bacteroidota, Proteobacteria, and Firmicutes, which was consistent with the dominant phylum found in the gut of *H. orientalis*, which is also a genus of medical leech (Whitaker et al., 2014). After blood meal feeding, the abundance of Bacteroidota increases, which has been shown to help the host efficiently absorb and utilize carbohydrates, proteins, and other substances (Murphy et al., 2010). The results of gut microbial studies on *Tachypleus tridentatus* (Miao, et al., 2020) after feeding also indicated that the abundance of Bacteroidota would show an increasing trend after ingestion, which was consistent with the findings of our research.

A report about the gut microbiota of captive *H. nipponica* showed that the main genera were *Aeromonas* and *Mucinivorans* after 14 days of feeding. However, for wild *H. nipponica,* the main genera in the gut were not only *Aeromonas* and *Mucinivorans* but also *Yokenella*. The difference in a growth environment and food sources causes this phenomenon, which was observed in our study. At the genus level, the relative abundances of *Aeromonas* and *Mucinivorans* increased significantly after feeding compared to before feeding. Interestingly, these two genera remained dominant until 30 days after feeding in our study. This result suggests that these two genera may act in the intestinal tract for more than 30 d after feeding. The two dominant genera were also found in the gut of leeches, such as *H. medicinalis* (Graf et al., 1999), *H. verbana* (Graf et al., 1999), and *M. decora* (Siddall et al., 2007). In addition, *Aeromonas* has been found in the digestive tract of blood-sucking animals such as *Culex quinquefasciatus* (Pidiyar et al., 2002). As a digestive tract symbiont, *Aeromonas* can secrete a hemolysin. This hemolysin releases glucose and potassium from erythrocytes, which leads to colloidal osmotic cleavage and hemoglobin release, achieving hemolysis (Howard et al., 1982; Stoebner et al., 1988) and aiding in the digestion of blood (Kikuchi and Graf 2007). As a parthenogenic anaerobic bacterium, *Aeromonas* can also inhibit the growth of other bacteria by reducing the local oxygen concentration (Worthen et al., 2006). *Mucinivorans*, as a new genus of *Rikenellaceae* (Nelson et al., 2015), is an obligate anaerobic bacterium. On the one hand, this bacterium participates in anaerobic metabolism and uses carbohydrates as carbon and energy sources (Marden et al., 2016) and can metabolize glucose, lactose, and other carbohydrates in the gut of the leech (Nelson et al., 2015) to provide energy for the host. On the other hand, this bacterium can also metabolize mucus produced in the gut after post-feeding, which likely provides it with an advantage in colonizing the digestive tracts of the leech (Marden et al., 2016). In the past, a report (Nelson et al., 2015) analyzed the whole genome of *Mucinivorans hirudinis which belong to Mucinivorans* in the gut of *H. verbana,* and found that the genomes are genes involved in cobalamin biosynthesis, which may be utilized to provide the leech host with vitamin B_{12}, which is deficient in vertebrate blood. Therefore, it is presumed that in *H. nipponia*, *M. hirudinis* may also provide vitamin B_{12}.

The relative abundance of genetic information processing (21.79%) and environmental information processing (15.37%) pathways increased significantly after ingestion, probably because the ingestion of blood meal caused *H. nipponia* to change from reliance on endogenous nutrients to consumption of exogenous nutrients and the gut microorganisms needed to adapt to the infusion of water and exogenous food from the environment. Therefore, the abundance of genetic and environmental information processing pathways increased. This result was consistent with the findings in the gut of *Siniperca chuatsi* (Chen et al., 2022). On the other hand, the abundance of nucleotide metabolism decreases, and abnormal nucleotide metabolism can cause a series of diseases. Excessively fast nucleotide metabolism can lead to excessively fast purine metabolism, eventually leading to the accumulation of uric acid, and excess uric acid can cause hyperuricemia (Zheng and Ma, 2016), which is harmful to the organism. The above results all indicate that ingestion is beneficial to improve the gut microbiota environment. Fresh porcine blood, as the food source in this study, contains a variety of proteins, as well as many amino acids and vitamins. Thus, after blood meal feeding, the abundance of pathways related to lipid metabolism, cofactor, vitamin metabolism, and exogenous substance degradation and metabolism increased, which may be related to the bait composition. In turn, the results of the prediction of gut microbial function in *Siniperca chuatsi* also indicate that different feed ingredients lead to changes in the relative abundance of each metabolic pathway (Chen et al., 2022).

Conclusion

Our study showed that the gut microbiota of *H. nipponica* before and after ingestion of blood meal had a lower structural similarity of intestinal flora and decreased richness. At the same time, two genera with high increased abundance are *Aeromonas* and *Mucinivorans.* Further exploration of its function can contribute to understanding the reasons for the variation in the relative abundance of the leech gut microbiota. Thus, it is important to study the gut microbiota and the influential mechanism of host physiological function.

Acknowledgments

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