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High-Throughput Sequencing Reveals the Effect of Feeding on Overwintering *Hirudo nipponia*

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Abstract: To investigate the effect of feeding on hibernating *Hirudo nipponia*, metagenomic sequencing was performed on the microorganisms collected from the digestive tract of *H. nipponia* individuals that were fed (FW) or starving (SW) before hibernation. In addition, the salivary gland tissues were obtained from these individuals to conduct transcriptome sequencing. Results showed that feeding before hibernation increased the relative abundance of Bacteroidetes and Proteobacteria at the phylum level and that of *Aeromonas*, *Mucinivorans*, *Bacteroides*, and *Desulfovibrio* at the genus level. Functional prediction results indicated that feeding before hibernation not only improved metabolic capacity but also increased the susceptibility of *H. nipponia* to pathogens. Among the 133,082 unigenes obtained by transcriptome sequencing, a total of 2801 differentially expressed genes (DEGs) were found, of which 355 were up-regulated and 2446 were down-regulated; the expression patterns of 15 selected DEGs were validated by qRT-PCR. Results from the GO and KEGG enrichment analyses showed that a majority of these DEGs were involved in the signal transduction pathways. This study established a basis for *H. nipponia* breeding during hibernation through analyses of the abundance of microorganisms in the digestive tract and the gene expression pattern of salivary glands by high-throughput sequencing. The findings also revealed the effect of feeding on the expression of both gastrointestinal microorganisms and salivary gland genes of hibernating *H. nipponia*.

Keywords: *Hirudo nipponia*; hibernation; metagenome; transcriptome; microorganisms in the digestive tract; salivary gland

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1. Introduction

Hirudo nipponia, belonging to the Hirudinidae family, prefers to live in dark and wet environments such as ponds, ditches, and swamps [1], and mainly feeds on the blood of livestock [2,3]. *H. nipponia* is one of the most recognized Chinese medicinal leeches and exhibits high anticoagulant activity [4]. The various anticoagulant-related active substances in the salivary gland of *H. nipponia* play a role in direct coagulation, analgesia, anti-inflammatory, blood flow promotion, inhibition of platelet aggregation, and degradation of exosomes during blood sucking [5]. Moreover, these active substances, such as hirudin [6,7], antistasin [8], hirustasin [9], and guamerin [10], as well as the newly found protein NPL-1 that has anticoagulant activity [11], further establish high medicinal value of *H. nipponia*. However, the wild resources of *H. nipponia* have been largely decreased due to deterioration of the environment and human activities, and artificial breeding of *H. nipponia* is a major way for maintaining the *H. nipponia* resources at present [12]. Therefore, researchers are actively exploring ways to increase the survival rate of leeches under artificial conditions [13].

Microorganisms inhabiting guts are closely related to food digestion and nutrition absorption, growth and development, and immune defense of animals [14,15], and can provide the best nutritional environment for the ingested food [16]. The gut microbes of blood-sucking leeches exhibit a strong symbiotic effect [17]; for example, *H. nipponia* gut microbes play an important role in the maintenance of vital signs during long-term

starvation [12]. These microbes help in obtaining nutrients by digesting and absorbing the sucked blood in the absence of corresponding enzymes [18,19], assist the decomposition of red blood cells, accelerate the rate of hemolysis, and maintain the integrity of the red blood cell structure, which can resist the invasion of pathogens and show a direct link with the immune response in the gut [20–22]. However, gut microbes can also become opportunistic pathogens or be manipulated by invasive pathogens to enhance infection and be detrimental to the host [23–26].

Hibernation is an adaptive behavior that helps animals reduce their energy expenditure during winter when food is scarce [27]. *H. nipponia* stays in deep hibernation at 2–12 °C [28], during which the gut microbes spontaneously alter the community structure to maintain a mutually beneficial relationship with the host under long-term fasting and low-temperature conditions [29,30]. In addition to changing the structure of gut microbial community, hibernation also affects the metabolic activity of gut microbes [31,32].

Illumina high-throughput sequencing technology is characterized by "sequencing-by-synthesis" [33], which not only reduces costs but also ensures high sequencing accuracy, showing advantages over the first-generation sequencing technology [34]. High-throughput sequencing technology has been widely applied in genomics, transcriptomics, and metagenomics research in humans, animals, and plants, as well as in microorganisms [35]. Metagenomics refers to the systematic study of metagenomes using non-culturing techniques and methods, that is, to study the community structure and ecological functions of microorganisms by analyzing the genome collection of microorganisms in a specific environment [36]. As a high-throughput metagenomic technique, marker-gene amplicon analysis shows the advantages of rapidity, simplicity, and cheapness [37]. The gut microbiome is dominated by bacteria, and amplicon sequencing is generally performed using 16S rRNA fragments of the bacterial genome. Traditional assays based on 16S rRNA genes cannot be utilized to analyze the metabolic potential of microbial communities; therefore, it would be beneficial to predict the functional capabilities of microbial communities based on marker-gene data [38]. In addition to macro-genomes, recent high-throughput sequencing studies of leeches have included transcriptome sequencing [39]. Transcriptomics studies the overall pattern of gene transcription and regulation of transcriptional activities at the RNA level [35]; it also determines the transcriptional structure of genes and analyzes the molecular mechanism based on changes in transcriptional expression levels [40]. Transcriptome sequencing technology is developed on the basis of high-throughput sequencing technology [41] and is characterized by high throughput, high accuracy, and low cost [42], which provides a new method for the research on leeches.

In studies of overwintering *H. nipponia*, it was found that the mortality rate of *H. nipponia* individuals that are mortality fed before hibernation is higher than that of *H. nipponia* individuals fasted before hibernation. The effect of feeding before hibernation on *H. nipponia* remains elusive. The salivary glands, an important tissue in the digestive tract of *H. nipponia*, secrete a variety of active substances, including antibacterial substances in addition to anticoagulant substances. Therefore, in this study, metagenome and transcriptome sequencing were used to study the microbial composition of digestive tracts and gene expression patterns in the salivary gland of *H. nipponia* fed and fasted before hibernation, respectively. The results provide a theoretical basis for the scientific feeding of *H. nipponia* during hibernation.

2. Materials and Methods

2.1. H. nipponia Material and Culture Conditions

H. nipponia individuals used in this study were purchased from the Daweijia Liwen Aquaculture Base in Jinzhou District, Dalian City, Liaoning Province, China. One hundred of *H. nipponia* individuals in good conditions, with no scars on the surface and similar sizes, were selected. The initial weight of *H. nipponia* individuals was 0.46 ± 0.12 g. They were divided into two groups after feeding and cultured in two identical plastic boxes (248 × 180 × 150 mm). The light/dark cycle was 10/14 h for the first 30 d, and the leeches

were kept in the dark for the following 30 d; the water was changed every other day. *H. nipponia* individuals were placed into an incubator (MODEL SPX-150B-Z, Boxun, Shanghai, China) for stepwise cooling treatment. One group was fed on fresh chicken blood poured into the sterilized casing every 7 d for the first 30 d (denoted as FW), and the other group was starved (denoted as SW). Three biological replicates were set for each treatment group for metagenome sequencing, and salivary gland tissues were collected from 10 randomly selected *H. nipponia* individuals in each group for transcriptome sequencing.

To mimic the hibernation environment of *H. nipponia*, the initial temperature of the incubator was set at 25 °C, decreased by 4 °C every 5 d, maintained at 9 °C for 5 d, and finally decreased to 6 °C. *H. nipponia* lost the ability to feed at 6 °C, stopped moving, curled up into a crescent shape, and remained in the hibernation state for 30 d.

2.2. Sample Collection

To obtain the microbes in the digestive tract, the surface of the *H. nipponia* body was cleaned using sterile water, and the whole body was immediately frozen in liquid nitrogen. After the body recovered to a soft state, the surface of the *H. nipponia* body was sterilized with 75% ethanol, and *H. nipponia* was dissected to obtain the contents of the digestive tract as well as intestinal tissues, which were placed into 1.5 mL sterilized Eppendorf (EP) tubes and snap-frozen in liquid nitrogen. *H. nipponia* individuals were dissected along the abdomen starting at the mouth. The salivary glands were cut as accurately as possible using ophthalmic scissors, placed in 1.5 mL EP tubes, and snap-frozen in liquid nitrogen. All samples were stored at −80 °C until further use.

2.3. DNA Extraction and Metagenomic Library Preparation

DNA was extracted using E.Z.N.A Mag-Bind Soil DNA Kit (M5635-02, Omega Bio-Tek, Norcross, GA, USA), DNA integrity was detected by 1.5% agarose gel electrophoresis, and DNA concentration was determined by a Qubit 3.0 fluorometer (Thermo Fisher Scientific, Carlsbad, CA, USA). Metagenome sequencing was performed by Sangon Bioengineering Co., Ltd. (Shanghai, China) based on an Illumina MiSeq platform. The primer pair 341F/805R (341F: 5′-CCTACGGGNGGCWGCAG-3′, 805R: 5′-GACTACHVGGGTATCTAATCC-3′) was designed according to the conserved sequence of the V3-V4 region of bacterial 16S rDNA. After the first round of PCR amplification, the Illumina bridge PCR compatible primers were introduced for the second round of amplification using 2× Hieff Robust PCR Master Mix (10105ES03, Yeasen Biotech Co., Ltd., Shanghai, China). The library size was detected by 2% agarose gel electrophoresis, and the concentration was determined using a Qubit 3.0 fluorometer (Thermo Fisher Scientific, Carlsbad, CA, USA).

2.4. Analysis of Metagenomic Data

The adaptors were removed from the raw paired-end reads, which were then filtered according to the following criteria: the bases with a quality score below 20 at the tail end were excised using PRINSEQ; if the average quality score in a 10 bp window was below 20, the back-end bases were truncated from the window. The N-containing reads and short sequences were removed, and the low-complexity sequences were also filtered out. Operational taxonomic unit (OTU) clustering and taxonomic identification were performed based on clean reads. Alpha-diversity analysis, beta-diversity analysis, and sequencing depth were investigated based on OTU clustering results, and community structure was examined at various taxonomic levels. Beta-diversity analysis based on Bray–Curtis distance, significance test, and functional prediction were performed, and independent *t*-test was conducted to analyze the functional differences of gut microorganisms by using SPSS 20.0 (IBM, Armonk, NY, USA). Beta-diversity patterns were visualized using the principal component analysis (PCA), principal co-ordinates analysis (PCoA) and nonmetric multidimensional scaling (NMDS).

We used PICRUSt (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States) and BugBase to predict functional changes in leeches. The PICRUSt

method was proposed to predict KEGG Ortholog (KO) using 16S rRNA gene sequence for microbial community function analysis, and 16S rRNA gene sequencing data were associated with microbial reference gene bank with known metabolic function. BugBase analyzes the phenotype of microbiome samples to help better understand the relationship between microorganisms and disease.

2.5. RNA Extraction and cDNA Library Preparation for Transcriptome Sequencing

Total RNA was extracted by FastPure Cell/Tissue Total RNA Isolation Kit (RC101, Vazyme Biotech Co., Ltd., Nanjing, China), and RNA concentration and integrity were determined by a Qubit 2.0 fluorometer (Thermo Fisher Scientific, Carlsbad, CA, USA) and agarose gel electrophoresis, respectively. cDNA library construction was performed by Sangon Bioengineering Co., Ltd. (Shanghai, China) and sequenced on an Illumina HiSeq 2500 platform.

2.6. Analysis of Transcriptome Data

Raw sequencing data were quality controlled using the FastQC online tool. Data processing was performed using Trimmomatic to filter the raw data for sequences with connectors and low-quality sequences in the raw data to obtain clean data. The clean data were De novo assembled into transcripts using Trinity and the transcripts obtained from Trinity assembly were de-redundant and the longest transcripts in each transcript cluster was taken as the unigene, which was used as the reference sequence for subsequent analysis. The transcripts were compared with the CDD (https://www.ncbi.nlm.nih.gov/cdd/ (accessed on 1 November 2021)), KOG (https://www.ncbi.nlm.nih.gov/COG/ (accessed on 1 November 2021)), COG (https://www.ncbi.nlm.nih.gov/COG/ (accessed on 1 November 2021)), NR (http://ncbi.nlm.nih.gov/ (accessed on 1 November 2021)), NT (http://ncbi.nlm.nih.gov/ (accessed on 1 November 2021)), PFAM (http://pfam.xfam.org/ (accessed on 1 November 2021)), SWISS-PROT (http://www.uniprot.org/ (accessed on 24 November 2021)), and TrEMBL (http://www.uniprot.org/ (accessed on 24 November 2021)) databases using NCBI BLAST+, and the functional annotation information was obtained.

The method took into account the effect of sequencing depth and gene length, as well as the effect of the sample on reads count, by computing TPM (transcripts per million) values to compare gene expression differences between samples. TMM was used to standardize read count data, followed by DEGseq for differential analysis. Significant differential genes were obtained using the following criteria: the TPM value ≥ 5 in at least one of the two samples; $|\log2(\text{fold-change})| > 1$; and Q-value < 0.05. Cluster Profiler was used for KEGG pathway and KOG classification enrichment analysis of differential genes. Q value < 0.05 indicates significant enrichment of this function.

2.7. qRT-PCR Analysis

Fifteen differentially expressed genes (DEGs) were selected from cAMP signaling pathway and cGMP-PKG signaling pathway for qRT-PCR analysis, using β-actin as an internal reference gene. Specific primers were designed according to the cDNA sequences using Clone Manager software (Table S1). After extracting total RNA from the same sample for transcriptome sequencing, first-strand cDNA was synthesized using the HiScript III RT SuperMix for qPCR kit (R323, Vazyme Biotech Co., Ltd., Nanjing, China), which completely removes residual genomic DNA from RNA templates. Subsequently, the qPCR system was configured according to the instructions of the PerfectStart^{TM} Green qPCR SuperMix kit (AQ601, TransGen Biotech Co., Ltd., Beijing, China), and qRT-PCR was performed on an ABI Prism7500 fluorescence qPCR instrument. The reaction volume was 10 μL, including 3.4 μL of ddH_2O, 0.3 μL of each primer, 5 μL of 2× PerfectStart Green qPCR SuperMix, and 1 μL of cDNA template (5 ng. μL^{-1}). The reaction was carried out according to the two-step qPCR method: pre-denaturation at 95 °C for 30 s; and 40 cycles of denaturation at 95 °C for 5 s, annealing at 60 °C for 34 s.

3. Results

3.1. Microbial Diversity in the Digestive Tracts of H. nipponia

A total of 265,253 raw reads were obtained from six samples, and 263,306 clean reads were obtained after quality control. Clustering analysis yielded 134 OTUs, with a coverage of >99.9% for both FW and SW groups (Table 1). Results of alpha-diversity analysis showed that the Shannon, Chao1, and Ace indices of the FW group were all higher than those of the SW group (Table 1), indicating higher microbial richness of the FW group compared with the SW group. A clear distance between the FW and SW groups was observed in both PCA (Figure 1C) and PCoA (Figure 1D) at the OTU level, suggesting the microbial community structures were different between these two groups. Moreover, the NMDS analysis at the OTU level also indicated differences in the non-linear structure of microbial communities between the two groups, with a stress value of <0.05 (Figure 1E). The results of Venn diagrams showed that the total number of OTUs shared between these two groups was 111, with 12 and 11 OTUs specific to the FW and SW groups, respectively (Figure 1B).

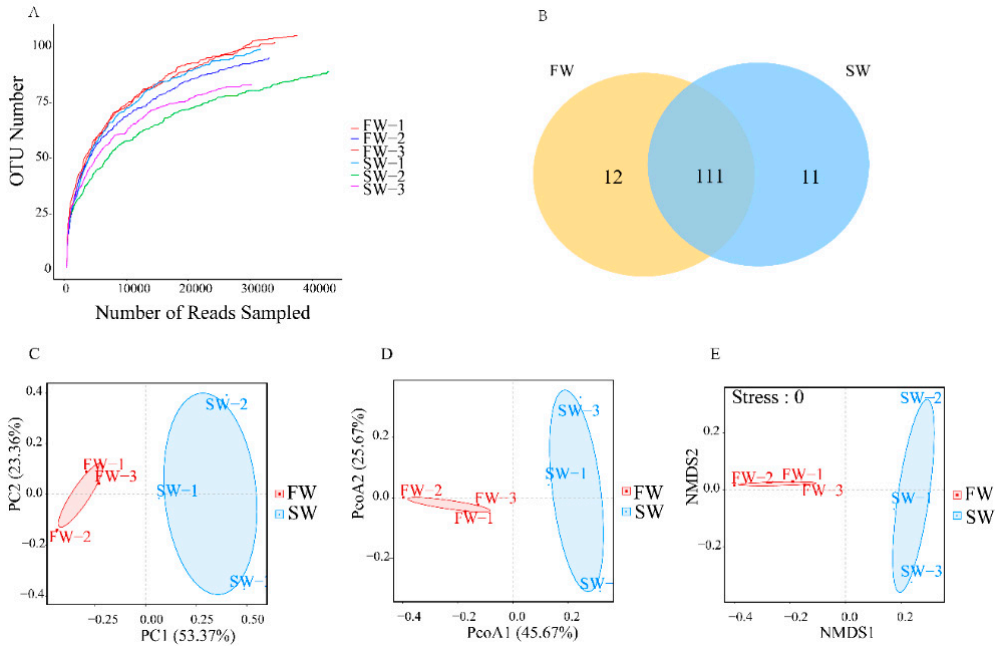

Figure 1. Compositional variation in microbial communities between FW leeches and SW leeches. (**A**) Alpha-diversity rarefaction plot of fecal microbiotas between FW leeches and SW leeches. The X- and Y-axes represent sample size and number of observed OTUs, respectively. (**B**) Venn diagram of OTU in FW leeches and SW leeches. Venn diagram summarizing the overlap of OTUs of leech fecal microbiota in FW leeches and SW leeches. (**C**) Principal Component Analysis (PCA) of bacterial diversity of FW leeches and SW leeches. (**D**) Principal co-ordinates analysis (PCoA) of bacterial diversity based on the Bray–Curtis distance of OTUs of FW leeches and SW leeches. (**E**) Non-metric Multidimensional Scaling (NMDS) ordination between microbial communities of FW leeches and SW leeches. Each point represents an individual leech sample.

Table 1. Phylogenetic diversity indices of FW and SW. Sequence coverage bacterial diversity indices: Chao1, Ace, and Shannon index in leeches.

Sample	Chao1	Ace	Shannon	Coverage
FW	114.21 ± 6.83	120.82 ± 6.14	1.75 ± 0.31	0.99 ± 0.00
SW	105.86 ± 13.31	105.98 ± 11.24	1.48 ± 0.16	0.99 ± 0.00

Values are means ± SD. Independent-sample *t*-test, $n = 3$ in each group.

3.2. Changes in Microbial Abundance in the Digestive Tract of *H. nipponia*

Figure 2 shows that Bacteroidetes, Firmicutes, and Proteobacteria were dominant phyla in the digestive tract of *H. nipponia* from both the FW and SW groups, accounting for more than 99% of the total microbes. The relative abundance of Bacteroidetes and Proteobacteria was higher in the FW group compared with the SW group, while that of Firmicutes was lower. At the genus level, the top three dominant genera in the digestive tract of *H. nipponia* were different between the two groups, with an order from high to low abundance being *Bacteroides*, *Hydrogenoanaerobacterium*, and *Mucinivorans* in the FW group, and that being *Hydrogenoanaerobacterium*, unclassified_Porphyromonadaceae, and unclassified_Ruminococcaceae in the SW group. In addition, the relative abundance of *Aeromonas*, *Mucinivorans*, *Bacteroides*, *Desulfovibrio*, and unclassified_Ruminococcaceae was higher in the FW group than in the SW group (Table 2).

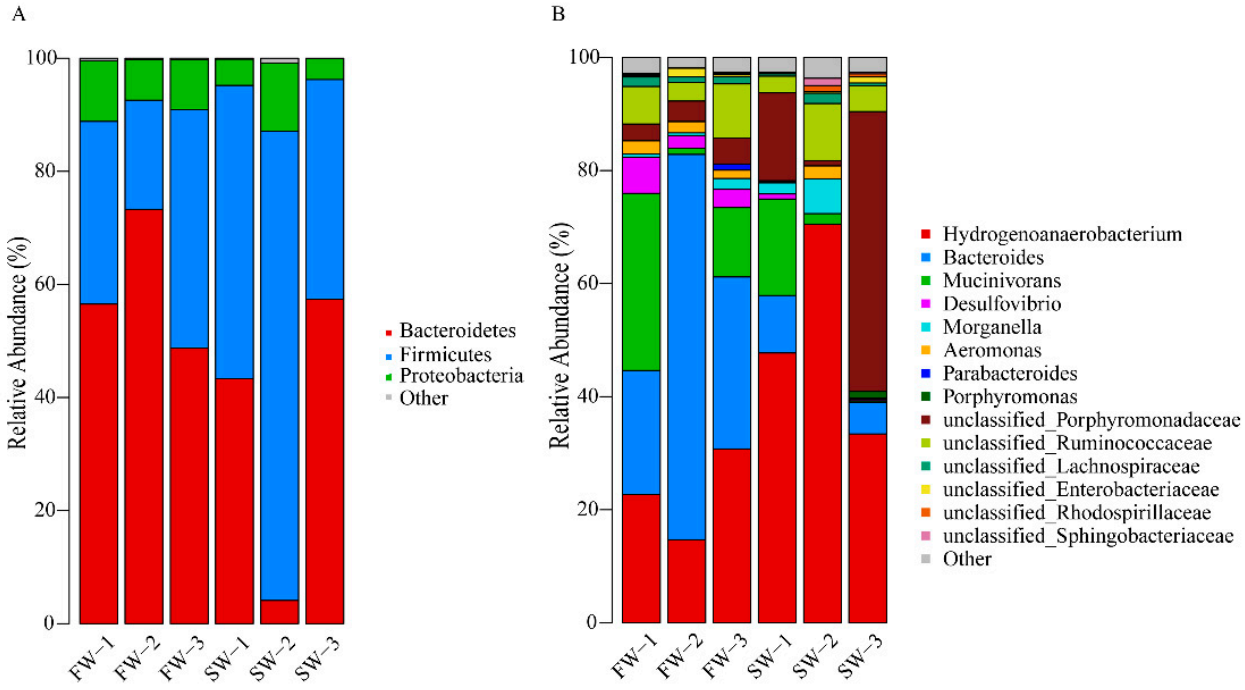

Figure 2. (**A**) Microbial community bar plot on the phylum level. Relative abundance of bacterial genera in each sample. (**B**) Microbial community barplot on the genus level. Relative abundance of bacterial genera in each sample.

Table 2. Relative abundance of dominant phyla in FW and SW.

OTUs	FW	SW	FW vs. SW
Bacteroidetes	59.52 ± 12.53	34.92 ± 27.61	Up
Firmicutes	31.25 ± 11.46	57.93 ± 22.70	Down
Proteobacteria	8.93 ± 1.78	6.78 ± 4.57	Up
Hydrogenoanaerobacterium	22.71 ± 8.04	50.54 ± 18.69	down
Mucinivorans	14.90 ± 15.30	6.40 ± 9.26	up
Bacteroides	40.17 ± 24.61	5.25 ± 5.06	up
Desulfovibrio	3.96 ± 2.16	0.38 ± 0.46	up
Aeromonas	1.93 ± 0.45	0.84 ± 1.23	up
unclassified_Porphyromonadaceae	3.72 ± 0.86	21.98 ± 24.95	down
unclassified_Ruminococcaceae	6.51 ± 3.13	5.87 ± 3.76	up

Values are means ± SD, $n = 3$ in each group.

3.3. Functional Prediction of Leeches

Based on the metagenome from our 16S rRNA sequencing results, PICRUSt and BugBase were employed to predict the metabolic function of microbes in the digestive

tract of *H. nipponia* and its relationship with diseases. KEGG enrichment analysis showed that compared with the SW group, the FW group exhibited significant enhanced activities in 'amino acid metabolism', 'nucleotide metabolism', 'lipid metabolism', and 'infectious diseases' categories ($p < 0.05$), while decreased activities in 'membrane transport', 'cellular processes and signaling', and 'cell motility' (Table 3). In addition, according to the BugBase analysis, the abundance of microbes related to 'potentially pathogenic' was higher in the FW group relative to the SW group (Figure 3A). These results suggest that feeding before hibernation not only altered the metabolic and cellular activities of *H. nipponia* during hibernation but also played an important role in the immune system and pathogen invasion activities.

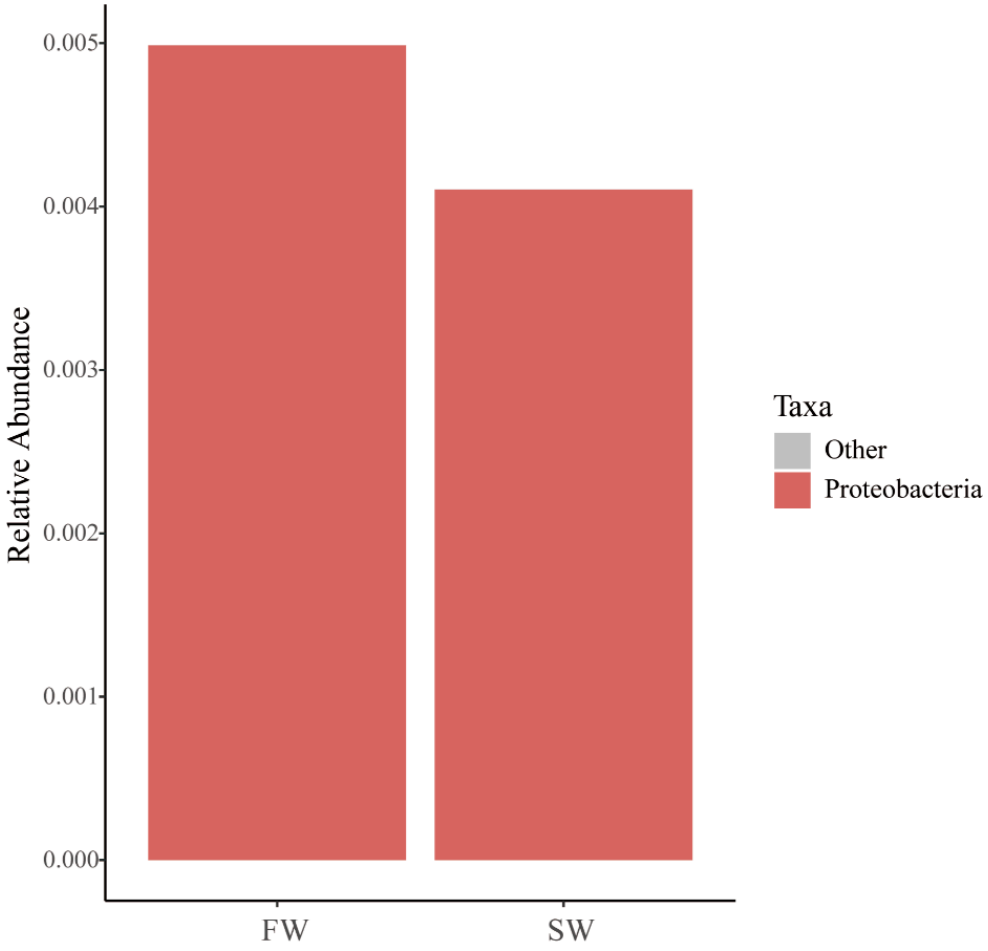

Figure 3. BugBase showing predicted relative abundance of *Potentially_Pathogenic* in FW leeches and SW leeches.

Table 3. PICRUSt showing predicted relative abundance of KEGG ortholog groups (Level 2 KOs).

CategoryL2	FW	SW	*t*	*p*
Membrane Transport	11.6526 ± 1.1476	14.451 ± 0.8439	−3.403	0.027
Carbohydrate Metabolism	10.6905 ± 0.1332	11.0392 ± 0.1289	−3.257	0.031
Amino Acid Metabolism	9.6914 ± 0.3057	8.9745 ± 0.1936	3.431	0.027
Poorly Characterized	5.1011 ± 0.0733	4.9643 ± 0.0246	3.063	0.038
Metabolism of Cofactors and Vitamins	4.2304 ± 0.1063	3.8005 ± 0.0944	3.704	0.021
Nucleotide Metabolism	4.1021 ± 0.0956	3.8637 ± 0.1097	2.838	0.047
Cellular Processes and Signaling	4.1819 ± 0.0610	4.3046 ± 0.0391	−2.94	0.042
Glycan Biosynthesis and Metabolism	2.3785 ± 0.3398	1.4765 ± 0.2654	3.624	0.022

Table 3. *Cont.*

CategoryL2	FW	SW	*t*	*p*
Lipid Metabolism	2.5681 ± 0.0394	2.4725 ± 0.0022	4.2	0.014
Folding, Sorting and Degradation	2.5454 ± 0.1745	2.169 ± 0.0985	3.253	0.031
Genetic Information Processing	2.5558 ± 0.0449	2.432 ± 0.0366	3.701	0.021
Transcription	2.5205 ± 0.1004	2.7929 ± 0.1074	−3.21	0.033
Cell Motility	2.7142 ± 0.4425	3.6728 ± 0.2267	−3.34	0.029
Metabolism of Terpenoids and Polyketides	1.6484 ± 0.0425	1.5329 ± 0.0235	0.914	0.043
Xenobiotics Biodegradation and Metabolism	1.8792 ± 0.0965	2.2023 ± 0.1472	−3.18	0.034
Metabolism of Other Amino Acids	1.3821 ± 0.1269	1.0696 ± 0.0516	3.949	0.017
Biosynthesis of Other Secondary Metabolites	0.9477 ± 0.0719	0.7393 ± 0.1480	3.747	0.02
Cell Growth and Death	0.4773 ± 0.0180	0.4328 ± 0.0110	3.656	0.022
Transport and Catabolism	0.3407 ± 0.0835	0.1277 ± 0.0532	3.729	0.02
Infectious Diseases	0.3891 ± 0.0175	0.3525 ± 0.0072	3.358	0.028
Signaling Molecules and Interaction	0.2137 ± 0.0417	0.1411 ± 0.0155	2.83	0.047

Values are means ± SD. Independent-sample *t*-test, $p < 0.05$, $n = 3$ in each group.

3.4. Identification of Differentially Expressed Genes (DEGs)

Transcriptome sequencing was performed on the salivary gland of *H. nipponia* from both the FW and SW groups, and 59,119,968 and 4,541,8088 raw reads were obtained, respectively. The proportion of reads with Q20 bases yielded from both groups exceeded 96.99%. After quality control and filtering, a total of 57,361,528 and 43,641,780 clean reads were obtained, respectively (Table S2). These clean reads yielded 133,082 unigenes, with N50 and N90 sizes of 983 bp and 245 bp, respectively, and an average length of 612.99 bp (Table S3). All unigenes were annotated (Table S4) by blasting against the GO, KEGG, and NR databases. A total of 2801 DEGs were identified between the two groups, of which 355 genes were up-regulated and 2446 genes were down-regulated (Figure S1).

3.5. GO and KEGG Enrichment Analyses of DEGs

GO clustering analysis was performed on the DEGs identified between the two groups. A total of 1393 DEGs were annotated to 68 GO groups, including 21 groups belonging to the molecular function (MF) category, 19 groups belonging to the cellular component (CC) category, and 26 groups belonging to the biological process (BP) category. Most of the DEGs in MF belonged to 'binding' (35.74%) and 'catalytic activity' (19.89%); most DEGs in CC were assigned to 'cell part' (45.98%) and 'cell' (45.98%); and in BP, 'cellular process' (45.56%) was the most abundant subgroup, followed by 'biological regulation' (39.66%) (Figure 4).

A total of 724 DEGs were classified into 33 categories of metabolic pathways, including 'signal transduction', 'endocrine system', and 'transport and catabolism' (Figure 5), and most of these DEGs were down-regulated (Table S5). Results from the hypergeometric test on these pathways showed that the 724 DEGs were significantly enriched in 97 metabolic pathways, including cAMP signaling pathway (ko04024), cGMP-PKG signaling pathway (ko04022), and focal adhesion (ko04510) (Figure 6).

3.6. Expression Patterns of Genes Related to Anticoagulation and Antibacterial Activities

Anticoagulation-related genes were identified from the transcriptome data, including those coding for analgesic and anti-inflammatory components that can interfere with immune and inflammatory responses (e.g., antistasin, ghilanten, bdellin, eglin, guamerin, piguamerin, and hirustasin); those coding for extracellular matrix degradation components (e.g., hyaluronidase); those coding for fibrinogen inhibitor components (e.g., destabilase); and the gene coding for hirudin that can directly inhibit thrombin activity. In addition, other antibacterial-related genes were also identified, including those coding for Theromyzin and Hirudomacin (Hmc). Compared with the SW group, the expression levels of genes coding for bdellin-KL, guamerin, Theromyzin, destabilase, and hyaluronidase were higher in the FW group (Figure 7).

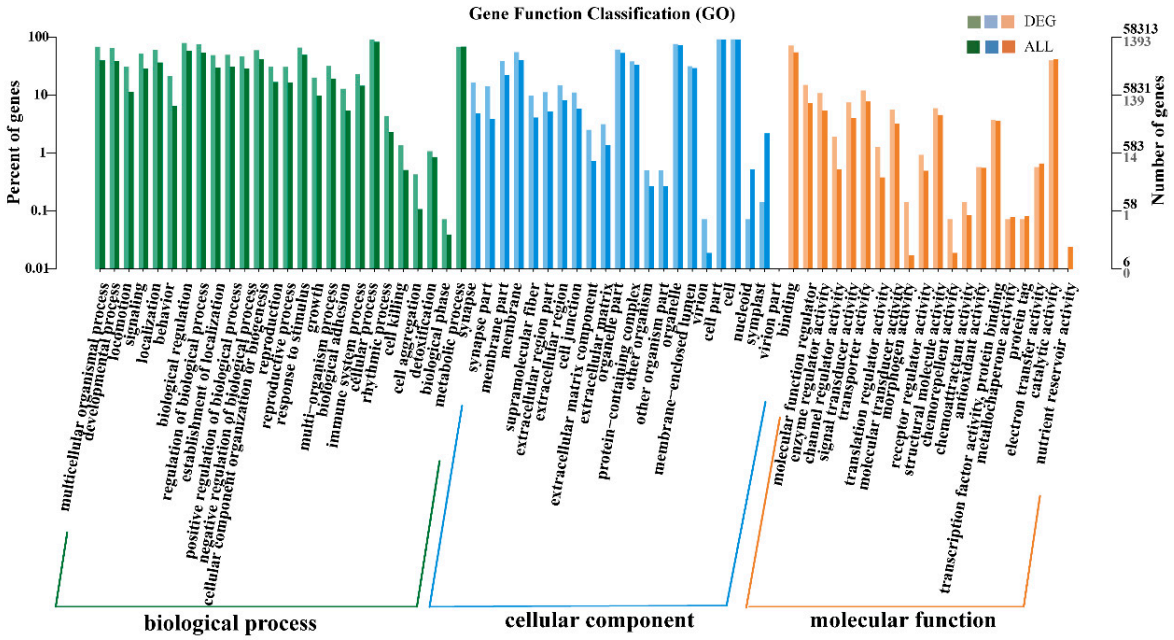

Figure 4. Gene Ontology (GO) functional classification of differentially expressed genes (DEGs) in FW leeches and SW leeches. Distribution of all-unigenes and DEGs among GO terms in biological processes, cellular component, and molecular function.

Figure 5. KEGG functional classification of differentially expressed genes (DEGs) in FW leeches and SW leeches.

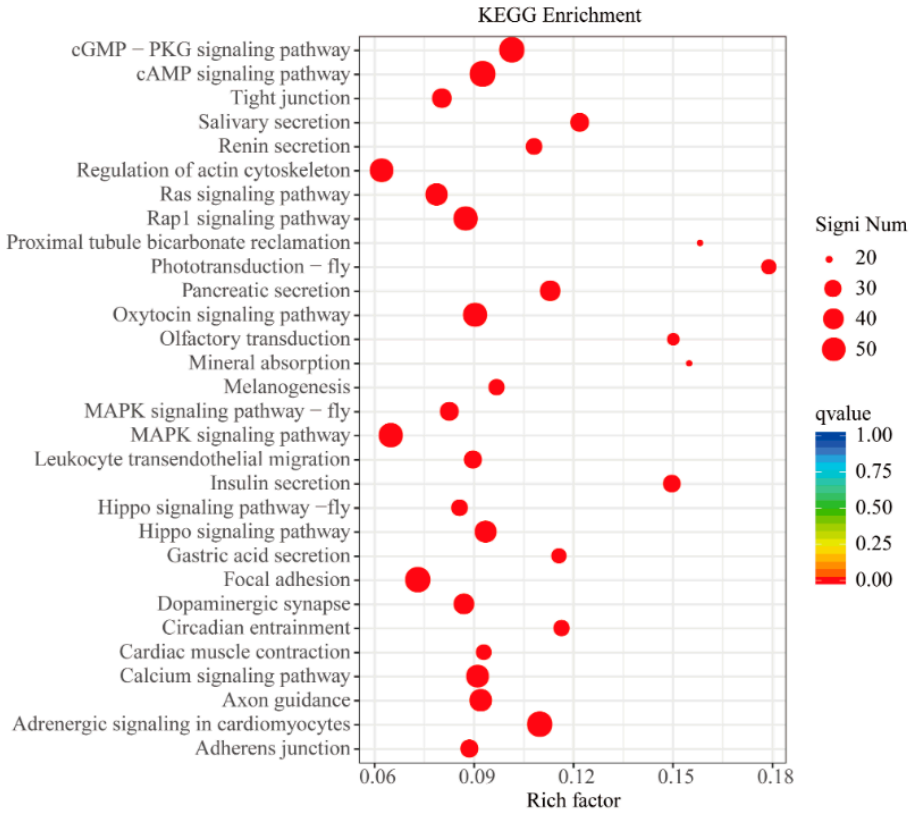

Figure 6. KEGG enrichment scatterplots of differentially expressed genes (DEGs) in FW leeches and SW leeches.

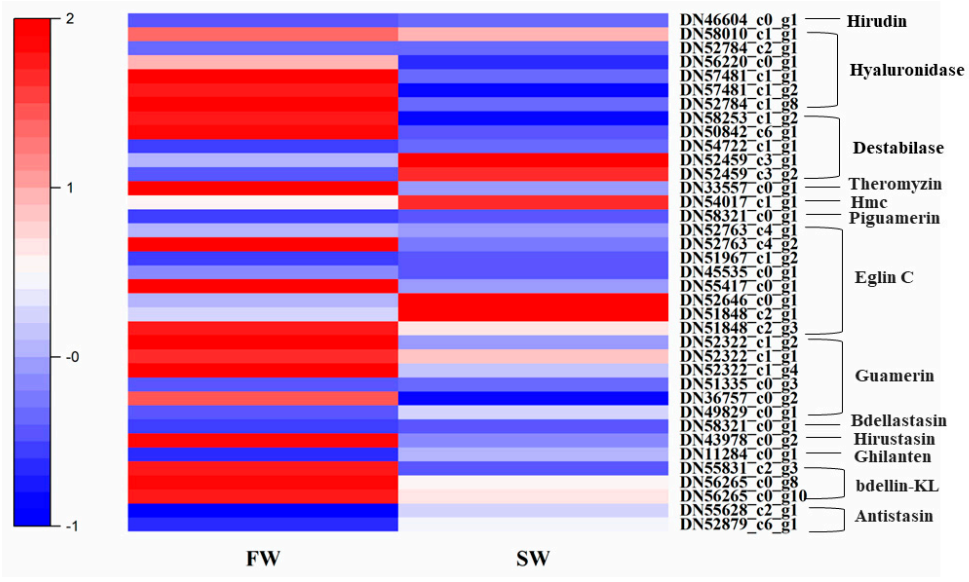

Figure 7. Heat map of gene expression related to anticoagulation and antibacterial activities in FW leeches and SW leeches.

3.7. qRT-PCR Validation

To verify the accuracy and reliability of transcriptome sequencing, 15 DEGs were selected for qRT-PCR validation. The expression patterns of all the 15 DEGs obtained from qRT-PCR analysis were consistent with those from transcriptome sequencing in both the FW and SW groups (Figure 8), indicating that the transcriptome sequencing results are reliable.

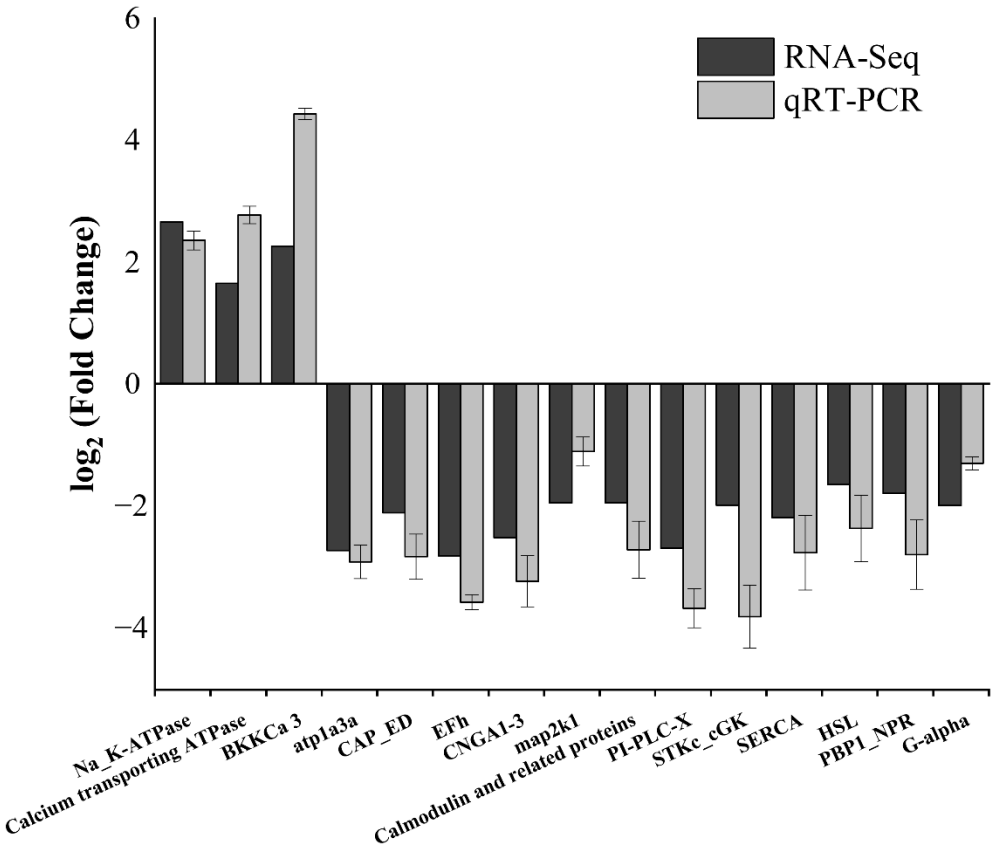

Figure 8. Comparison of the expressions of RNA-Seq and qRT-PCR results.

4. Discussion

To ingest and digest the host's blood, the salivary gland of *H. nipponia*, as part of the anterior segment of the digestive tract, can secrete more than 100 active substances [43,44], including many substances related to anticoagulation and antibacterial activities [41]. Microbes in the digestive tract aid in the digestion and absorption of sucked blood and the host immune response [15,18,21,22]. Metagenomic and transcriptome sequencing on the microorganisms in the digestive tract and salivary gland of *H. nipponia* were carried out in order to thoroughly explore the impact of feeding on overwintering *H. nipponia*. The results showed that feeding before hibernation altered the microbial community structure and increased the taxonomic richness in the digestive tract of *H. nipponia* (Table 1). Compared with the SW group, Bacteroidetes, Firmicutes, and Proteobacteria were the dominant phyla in the FW group, and the relative abundance of Bacteroidetes and Proteobacteria was higher. Bacteroidetes can promote carbohydrate fermentation and maintain the balance of gut microbiota [45]. In addition to participating in the metabolism of polysaccharides, bile acids, and steroids, Bacteroidetes also play a role in the metabolism of proteins and fats provided by the intestinal epidermis [46,47]. Proteobacteria are associated with the instability of gut microbiota [48] and are the main cause of the increase in the potential pathogenicity in leeches. The dominant phyla in the digestive tract of *H. nipponia* revealed in this study are consistent with the findings of Shi et al. [12,49], and similar to those found in grass carp [50] and Nile tilapia [51].

At the genus level, the relative abundance of *Mucinivorans*, *Bacteroides*, *Desulfovibrio*, *Aeromonas*, and unclassified_Ruminococcaceae was higher in the FW group relative to the SW group (Figure 2, Table 2). *Bacteroide* are widely distributed in humans, animals, and aquaculture environments. An increase in the abundance of *Bacteroide* indicates a pro-inflammatory response [52], which may also be one of the reasons for increased pathogenicity of leeches. *Mucinivorans* belonging to Rikenallaceae [16,53] is involved in

anaerobic metabolism and utilizes carbohydrates as an energy source [54]. It metabolizes glucose, lactose, mannose, and melibiose [55], as well as mucus secreted by enterocytes of leeches, providing favorable habitats for the colonization of gut microbiota [56]. *Desulfovibrio*, a kind of sulfate-reducing bacteria (SRB), does not produce toxins [57], but generates a large amount of H_2S in the process of reducing sulfate, which changes the environmental pH and inhibits the normal metabolism of aerobic bacteria [58]. It is associated with the occurrence and development of enteritis, intestinal cancer, and metabolic syndrome [59], and may be related to the antagonism between other microorganisms [49]. *Aeromonas* is a dominant genus in the gut of *H. medicinalis* [60,61], *Hirudo verbana* [16], and North American leech [62]. It is a Gram-negative facultative anaerobic bacterium, and *Aeromonas* species were reported as pathogens in most studies [63,64]. *Aeromonas* can secrete hemolysin, which can release glucose and potassium from erythrocytes, thereby enabling colloid osmotic lysis and hemoglobin release to achieve hemolysis [22,65] and helping blood digestion of *H. nipponia*. Ruminococcaceae can metabolize mucopolysaccharides and proteins [31,66], thereby assisting in digestion of food in the gut. The core genera in the digestive tract of *H. nipponia* revealed in this study are different from those found by Shi et al. [12,49], which is probably due to the different breeding and feeding conditions of *H. nipponia*.

Functional prediction of microbes in the digestive tract of *H. nipponia* based on PICRUSt analysis showed that the metabolic activities of amino acids, nucleotides, and glycans were significantly promoted in the FW group (Table 3). At the same time, the pathways associated with immune-related diseases were significantly increased. These results are consistent with those obtained from BugBase analysis in which increased pathogenicity was observed in the FW group (Figure 3). *H. nipponia* individuals fed before hibernation showed reduced immunity and were more susceptible to infection by pathogens, resulting in death during hibernation. The interactions between microbes altered with the changing microbial compositions, resulting in a change in the microbial functions in the digestive tract. We speculated that feeding before hibernation may potentially affect primary immune deficiencies and increase the risk of pathogen infection while altering the microbial community structure in the digestive tract of *H. nipponia* to improve intestinal absorption and metabolism.

Transcriptome analysis was performed on salivary glands of *H. nipponia* in both the FW and SW groups, and the obtained data help us understand the differential gene expression in salivary glands between these two groups. The most enriched KEGG category was signal transduction, including 97 pathways, with the most significantly enriched pathway being the cAMP signaling pathway (Figures 5 and 6). The down-regulation of genes involved in signal transduction pathways was speculated to be related to the reduced response to external stimuli during *H. nipponia* hibernation when the food in the digestive tract was sufficient. The expression levels of 15 DEGs selected in the cAMP and cGMP-PKG signaling pathways showed the same trend in qRT-PCR and RNA-seq analyses, which verified the reliability of RNA-seq (Figure 8).

Leeches stimulate host bleeding through anticoagulant substances [16,18,67,68], and antibacterial active ingredients secreted by the salivary gland can inhibit the growth of ingested microbes when leeches ingest host's blood. At the digestive tract of leeches, the water in the blood is concentrated; the blood is stored in the foregut and digested and absorbed in the intestinal tract. The protein decomposition process in the leech gut requires a cooperation of multiple proteases that break down different peptide bonds [69]. The digestion process of proteins in blood cells in the leech gut is slow during hibernation, which is related to the fact that protease inhibitors can regulate exopeptidases and endopeptidases secreted by microbial symbionts in the leech gut [70]. The protease inhibitors secreted by the salivary gland, e.g., eglin and bdellin [69], are involved in the digestion and storage of blood in the gut. Eglin is a serine inhibitor, and eglin-C is classified as an anti-inflammatory agent due to its ability to effectively block the activities of neutrophil elastase and cathepsin G secreted by neutrophils [71], which can induce inflammatory defense responses. Bdellin can specifically inhibit trypsin, plasmin, and acrosome protease

chymotrypsin [72]. Both bdellin A and bdellin-KL are non-classical Kazal-type cysteine protease inhibitors, which exert anti-inflammatory effects by inhibiting proteases involved in the spread of inflammation [73]. Serine protease, as a proteolytic enzyme with a wide range of biological activities, participates in biological activities including food digestion and immune system regulation [74]. In addition to eglin, serine protease inhibitors secreted in leech salivary glands also include antistasin, ghilanten, piguamerin, guamerin, and hirustasin [75]. Antistasin and ghilanten are highly homologous and harbor very similar functions. They are both serine protease inhibitors and can effectively inhibit coagulation factor Xa [9,76]. Both guamerin and piguamerin can inhibit neutrophils and pancreatic elastase; however, guamerin is more specific in effectively inhibiting plasma kallikrein, tissue kallikrein, and pancreatic enzymes [77,78]. Hirustasin, with its strong anti-inflammatory effect, is the first tissue kallikrein inhibitor found in leeches, which can inhibit trypsin, chymotrypsin, and neutrophil cathepsin G [9,79].

Hyaluronidase has antibiotic and β-endo-glucuronidase activities and can digest hyaluronic acid in the extracellular matrix [80]. Theromyzin, the first anionic antimicrobial peptide observed in invertebrates, can inhibit the activity of Gram-positive bacteria [81]. Hirudomacin, secreted in both the gut and salivary gland, can lyse bacteria by affecting bacterial membranes through electrostatic interactions, and has significant antibacterial activity against Gram-negative and Gram-positive bacteria [20]. Destabilase is a multifunctional protein that not only inhibits fibrinogen, but also acts as lysozyme to lyse bacterial cells by digesting the β-1,4 glycosidic bond of peptidoglycan (a component of bacterial cell wall) or inhibits *Micrococcus luteus* and *Escherichia coli* by the peptide synthesized from the sequence of its α1 domain [82]. Although the proportion of pathogenic bacteria in gut microbes was up-regulated in *H. nipponia* fed before overwintering, which were outwardly more susceptible to mortality (Figure S2), gene expression in salivary glands also changed significantly. Significantly higher expression was observed in bdellin-KL, guamerin, theromyzin, destabilase, and hyaluronidase, which have anti-inflammatory and antibacterial effects (Figure 7), suggesting regulation by digestive tract microbes to cope with external microbes encountered in post-hibernation feeding and thus gut microbial instability.

Hirudin is a potent and specific thrombin inhibitor, which directly binds to thrombin [6,83]. The expression level of hirudin was low during the hibernation of *H. nipponia*, which was speculated to be related to the decreased demand for food during hibernation. When sucking blood, in addition to the fact that the salivary gland secretes a large number of anticoagulant and antibacterial active substances, the abundance of microbes in the leech gut also increases [20,84]. When the pathogenicity of microbes in the digestive tract increases, the expression of genes related to antimicrobial substances is also increased in salivary glands.

5. Conclusions

In this study, high-throughput sequencing was performed on microbes in the digestive tract and salivary gland of *H. nipponia* fed or fasted before hibernation, and the changes in microbial community structure in the digestive tract and the differential gene expression in the salivary gland during hibernation were analyzed. Feeding before hibernation not only increased the microbial diversity as well as the abundance of microbes related to metabolism and infectious diseases in the digestive tract of *H. nipponia*, but also promoted the expression of antibacterial-related genes in salivary glands. The stability of the microbes in the digestive tract of *H. nipponia* is jointly maintained by the salivary gland and the gut microbes, and the changes in the microbes in the digestive tract can lead to differential gene expression in the salivary gland. These results provide new insight for the research on the relationship between gene expression in salivary glands and the changes in bacterial composition in the digestive tract of *H. nipponia* and lay a foundation for the study of overwinter culture in *H. nipponia*. However, further investigations are needed on the relationship between gene regulation in salivary glands and microbes in digestive tracts.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/d14090768/s1, Table S1: Primers used for gene expression validation; Table S2: Statistic summary of sequencing data; Table S3: Statistic summary of De nove assembly data; Table S4: Statistics of functional annotation; Table S5: Gene expression in KEGG enrichment in FW and SW; Figure S1: Differential gene scatter diagram in FW leeches and SW leeches; Figure S2: Survival rate of FW leeches and SW leeches.

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