

Research Article

Response of Gut Microbiota, Digestive Enzyme Ability, and Immune Function to Starvation in the Oriental River Prawn *Macrobrachium nipponense*

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In order to evaluate the interplay of nutritional status and the digestive capacity, immune function, and gut microbiota in the oriental river prawn *Macrobrachium nipponense*, a 14-day starvation trial was conducted to detect the effects of starvation on the activities of digestive enzymes and immune enzymes and the structure of prawn gut microbiota. The adult prawns were randomly assigned to a control group (F group) and starvation group (S group) with three replicates. The F group was normally fed with commercial diet, and the S group was starved for 14 days. The result showed that digestive enzyme activities of trypsin and lipase in the hepatopancreas of the S group were significantly lower than those of the F group after 4 d of starvation ($P < 0.05$), while the activity of amylase significantly increased after 14 d of starvation ($P < 0.05$). Moreover, the results of the histological analysis of prawn gut showed that both the height of epithelial cells and microvilli of intestine in the S group were obviously decreased than those in the F group ($P < 0.05$) after 14 d of starvation. The activities of immune-related enzymes including superoxide dismutase, catalase, and acid phosphatase in the hepatopancreas of the S group significantly decreased after 4 d of starvation, and lysozyme activity of the F group was lower than that of the S group after 7 d of starvation ($P < 0.05$). In addition, the results of Illumina high-throughput sequencing showed that a total of 14,285 OTUs were obtained and classified into 30 phyla, among which Actinobacteria and Proteobacteria were the predominant microflora in the intestinal microbial communities of both groups. However, the relative abundance of opportunistic pathogens was significantly increased in the S group, while the relative abundance of beneficial bacterium was decreased ($P < 0.05$). The bacterial richness with the Chao estimator was significantly higher in the F group than in the S group ($P < 0.05$). Both the results of principal coordinate analysis (PCoA) and nonmetric multidimensional scaling (NMDS) demonstrated that the intestinal microbiota of the F group was separate from those of the S group. The result of functional prediction of the metabolic pathways showed that the intestinal microbiota was enriched in the KEGG pathways of amino acid, carbohydrate, fatty acid, and lipid biosynthesis and degradation at level 2. This result implied that the microbiota of shrimp gut decreased nutrition metabolism under the stress of starvation. Meanwhile, comparing to the F group, the immune-related pathway of enterobacterial common antigen biosynthesis was markedly reduced in the S group ($P < 0.05$). The result of redundancy analysis (RDA) further confirmed that the activities of digestive and immune enzymes were correlated with the microbial community structure. Finally, the structural equation modeling highlighted that changes in the activities of digestive and immune enzymes were directly related to the gut bacterial community and notably affected prawn growth.

1. Introduction

Aquaculture species suffer from transient food deprivation caused by climate changes, migration, and reproduction and may also suffer from starvation due to feed cost reduc-

tion, stress of transportation, management of disease outbreaks, and water quality amelioration [1]. During the starvation period, animal metabolism is adapted to utilize fuel stores for surviving and maintaining body functions [2, 3]. It was reported that shrimp could regulate the

TABLE 1: Growth performance of prawn under the treatment of starvation and normal feeding.

	Feeding group	Starvation group
W_0 (g)	2.89 ± 0.08	2.89 ± 0.08
W_t (g)	$3.32 \pm 0.11^*$	2.59 ± 0.16
WGR (%)	$15.32 \pm 0.37^*$	$-(10.12 \pm 1.00)$

Data are presented as mean \pm SD ($n = 10$). Statistical significance is tested using an unpaired t -test ($*P < 0.05$). W_0 : initial weight; W_t : final weight; WGR: weight growth rate.

digestion activity in response to short-term starvation [4]. The activities of the digestive enzymes could be significantly downregulated in shrimp during fasting periods, reducing the mechanochemical stimulators of the gastrointestinal tract [5] and drastically altering the morphological structure of the gastrointestinal tract [6]. The digestive enzyme activities of crustaceans under the stress of starvation depend on the duration of food deprivation. The protease activities in the juveniles of *Fenneropenaeus chinensis* were significantly decreased when exposed to food deprivation at different periods and then rapidly increased after food refeeding, but the changes of amylase and lipase activities exhibited opposite trends [7]. The activities of amylase, lipase, and pepsin were significantly lower in the starved group of *Litopenaeus vannamei* than in a normal fed group [8]. Because of the inconsistent results as mentioned above, it is necessary to further investigate the response of the digestive enzymes and their regulation mechanism under starvation stress in more shrimp species. Previous studies had demonstrated that starvation also affected the nonspecific immune activity of fishes. Starvation significantly promoted the lysozyme activities in the sea bass *Dicentrarchus labrax* and *Pagellus bogaraveo* [2] and the activities of antioxidative enzymes and such as superoxide dismutase (SOD) in *Labeo rohita* [9]. Nevertheless, little is known about the mechanism of starvation on immune activities of crustaceans. The intestinal microbes are a complex organ ecosystem and have a profound influence on nutrient metabolism, pathogen resistance, and immune system development [10]. Studies had found that the diet could shape the intestinal microbiota in the host [11, 12]. The changes in the gut microbial composition had marked effects on health, growth, and survival of farmed fishes and crustaceans [13]. However, the information on the relationship between feeding regimes and intestinal microbiota was still limited in crustaceans. Recent studies had shown that starvation stress affected the composition structure of intestinal microbiota [8], and the changes in the gut microbial community were related to digestive and immune enzyme activities, which subsequently affected the growth of *L. vannamei* [14].

The oriental river prawn *Macrobrachium nipponense* is a nutritional and economic species of the decapod crustacean throughout China, Japan, and Vietnam [15]. Previous studies had proven that there was complete compensatory growth in this species following 2 or 4 d of starvation [16]. During the period of starvation, the activities of pepsin, trypsin, and lipase significantly decreased while the activity of

amylase increased at 4 d, but the activities of immune-related enzymes SOD and CAT increased a little at 2 d and then decreased significantly at 8 d [5]. The effects of starvation on the composition of intestinal microbiota and the interplay among shrimp gut microbiota and digestive and immune enzyme activities in *M. nipponense* were still unclear. The aim of this study was to explore the effect of starvation stress on the changes and relationship among the activities of digestive and immune-related enzymes and the structure of gut microbiota in *M. nipponense*.

2. Materials and Methods

2.1. Rearing Procedures. All animal experiments were carried out under strict accordance with the Guide for the Care and Use of Laboratory Animal at Shaoxing University and were approved by the Bioethics Committee of Shaoxing University. Healthy prawns were obtained from a local freshwater farm in Shaoxing city (Zhejiang, China). Before the feeding trial, prawns were acclimated for one week and fed with commercial feed (Zhejiang Kesheng Feed Co., Ltd., Shaoxing, China) in round fiberglass tanks of 750 L. The composition of the feed (wet weight) contained 39.22% of crude protein, 6.25% of crude fat, 3.87% of crude fiber, 13.28% of crude ash, and 9.39% of moisture, and its gross energy was $4339 \text{ kcal kg}^{-1}$. At the end of the acclimatization period, a total of 180 prawns with an average initial weight of $2.90 \pm 0.08 \text{ g}$ and length of $6.31 \pm 0.11 \text{ cm}$ were randomly assigned to two groups with three replicates. The feeding group (F group) was continuously fed, and the starvation group (S group) was continuously fasted during the experimental period of 14 d.

2.2. Sample Preparation. At the end of the experiment, the weight growth rate (WGR) of the different groups was calculated according to the following equation: $\text{WGR (\%)} = 100 \times (W_t - W_0) / W_0$, where W_t is the weight of prawns at the termination of the experiment and W_0 is the weight of prawns at the start of the experiment.

At the end of the experiment period of 14 d, ten individual prawns per tank were randomly chosen and aseptically dissected in an ice bath; then, their intestinal tissues were isolated. Subsequently, the midguts of five prawns were segmented into the same length and fixed with 10% formalin solution for 24 h before histological analysis. The whole guts of the other five prawns were directly frozen in liquid nitrogen, then stored at -80°C before microbiota analysis. At 0, 4, 7, 11, and 14 d after starvation, the hepatopancreas from three prawns of both groups was immediately extracted and pooled for the measurement of the digestive and immune-related enzyme activities. These tissues were homogenized in 10x volumes (original tissue weight/volume) of phosphate buffer saline (PBS) and centrifuged at 6000 g for 10 min at 4°C . The supernatant was harvested to measure the activities of the digestive enzyme including amylase (AMS), trypsin (TRY), and lipase (LPS) and the immune-related enzyme including superoxide dismutase (SOD), catalase (CAT), lysozyme (LZM), and acid phosphatase (ACP) with the commercial kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China),

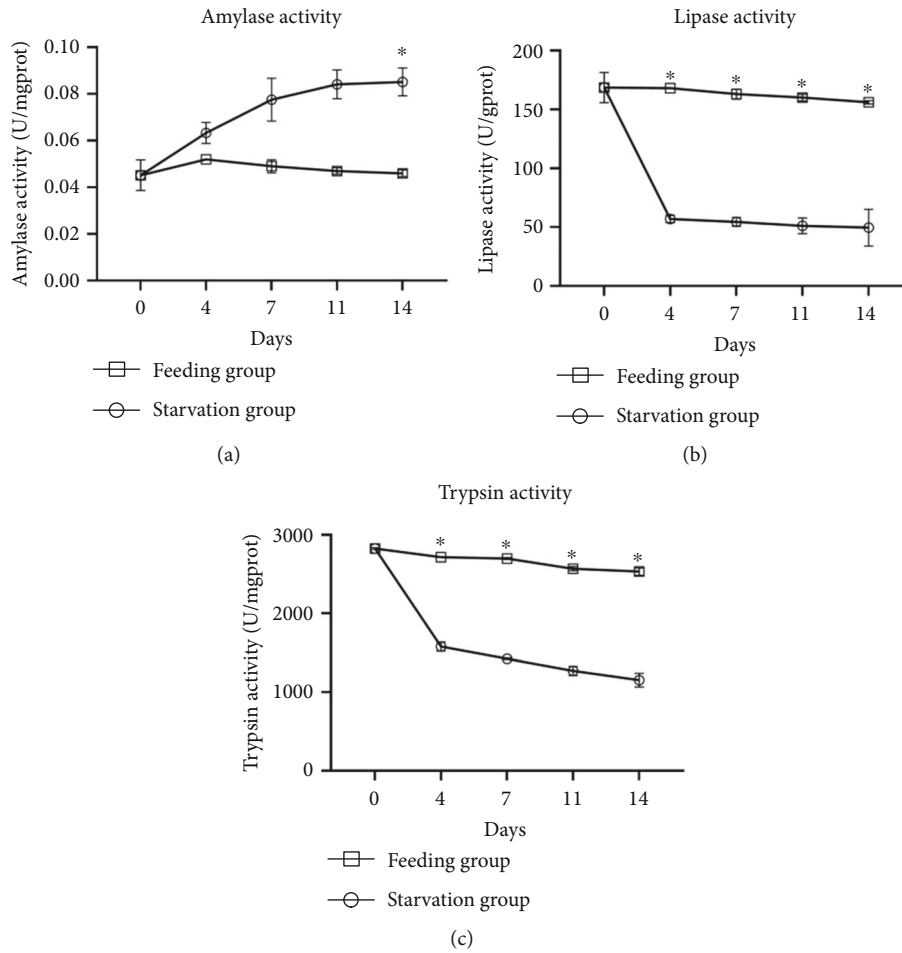


FIGURE 1: The changes of digestive enzyme activities in the hepatopancreas of *M. nipponense* after 14 d of starvation: (a) amylase (AMS); (b) lipase (LPS); (c) trypsin (TRY). Data are presented as mean \pm SD ($n = 6$). Statistical significance is tested using an unpaired *t*-test ($*P < 0.05$).

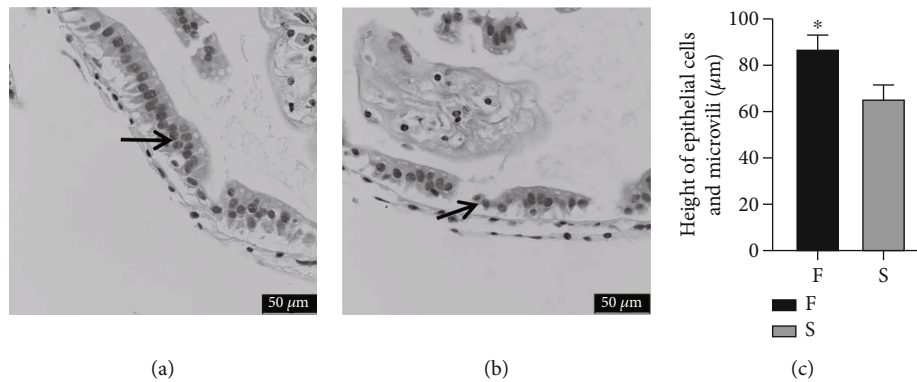


FIGURE 2: The changes of the intestinal histological structure in *M. nipponense* after 14 d of starvation by hematoxylin and eosin staining. Magnification is 400x, and the scale represents 50 μm . (a) Arrow shows that the epithelial cells were closely connected in the feeding group. (b) Arrow shows that the epithelial cells were shrunken or even disappeared in the starvation group. (c) Comparisons of the height of intestinal epithelial cells and microvilli in F (feeding) group and S (starvation) group. Results are presented as means \pm SD of ten observations for three samples in each groups. Statistical significance is tested using an unpaired *t*-test ($*P < 0.05$).

according to the manufacturers' protocol. Total protein concentration was determined by the method of Coomassie Brilliant Blue G-250 with bovine serum albumin as a standard [17]. The enzyme-specific activities were expressed as U mg^{-1} protein.

2.3. Digestive and Immune Enzyme Activity Assays. Amylase activity (U mg^{-1} protein) was determined by the iodine-starch colorimetric method and defined as hydrolyzing 10 mg of starch with 1 mg tissue protein acting on the substrate within 30 min at 37°C. Lipase activity (U mg^{-1} protein)

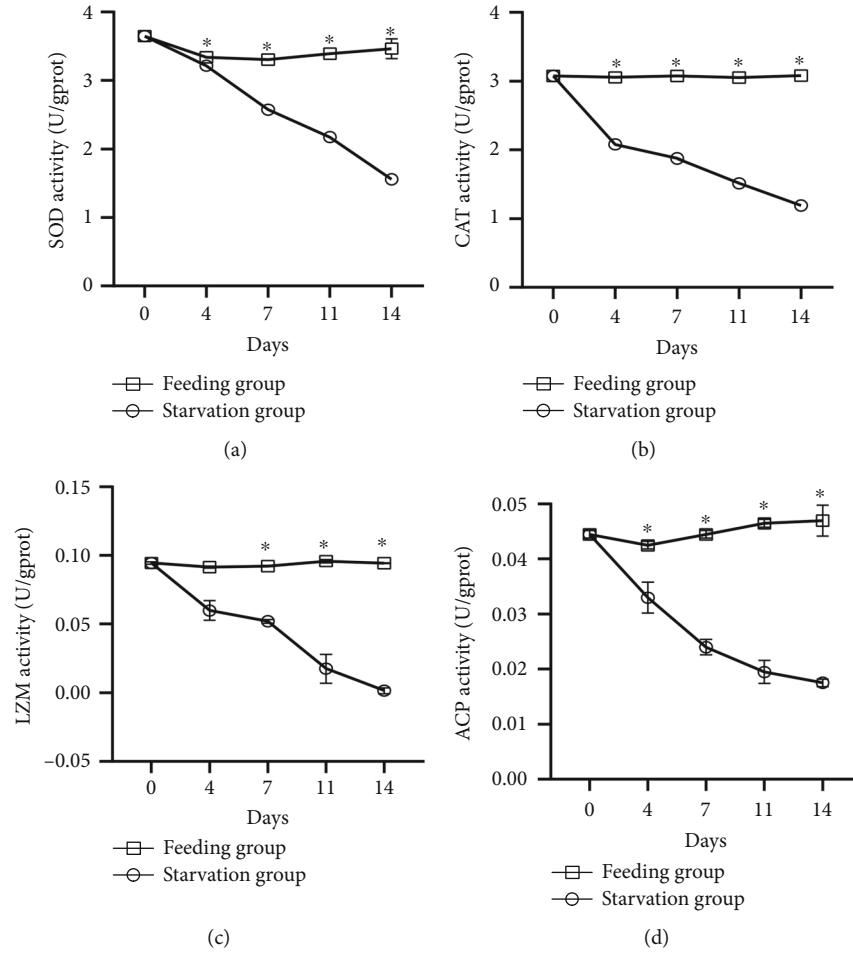


FIGURE 3: The changes of immune enzyme activities in the hepatopancreas of *M. nipponense* after 14 d of starvation: (a) superoxide dismutase (SOD); (b) catalase (CAT); (c) lysozyme (LZM); (d) acid phosphatase (ACP). Data are presented as mean \pm SD ($n = 6$). Statistical significance is tested using an unpaired *t*-test ($*P < 0.05$).

TABLE 2: Sequencing, OTU classification information, and Good's coverage in six samples.

Sample	Reads	Phylum	Class	Order	Family	Genus	Species	Unclassified	Total	Good's coverage
S1	62676	30	155	644	509	548	129	1	2081	0.994
S2	54168	21	171	487	559	632	182	0	2103	0.996
S3	56139	21	99	205	446	569	173	2	1569	0.997
F1	50795	13	230	1166	1030	539	108	0	3125	0.994
F2	53041	13	294	1012	701	565	115	0	2761	0.992
F3	61960	24	218	947	697	544	121	1	2656	0.989

was defined as the amount of enzyme which consumes $1 \mu\text{mol}$ substrate when incubating with 1 mg tissue protein in 1 min at 37°C . Trypsin was measured by incubating the mixtures in a water bath at 37°C , and absorbance A1 and A2 were recorded at 253 nm after mixing for 0.5 min and 20.5 min , respectively. The ACP activities were measured using a disodium phenyl phosphate method [18]. One unit of ACP activity was defined as the production of 1 mg total protein to produce 1 mg phenol in 15 min at 37°C . The SOD activity was measured spectrophotometrically using the xanthine oxidase-cytochrome c method at the wavelength of 550 nm [19]. One unit of SOD activity was defined

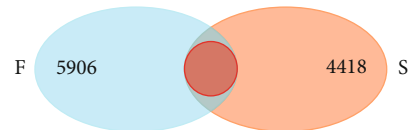


FIGURE 4: The distribution difference of the OTUs in the gut microbiota of the F group and the S group of *M. nipponense* after 14 d of starvation by Venn diagram.

as the amount required to inhibit the rate of reduction of cytochrome c by 50% and determined as ng of SOD mg^{-1} protein in the supernatant fraction. The CAT activity was

TABLE 3: Data from Illumina high-throughput sequencing yields bacterial diversity and richness based on operational taxonomic units (OTU), estimated OTU richness (Chao), and diversity index (Shannon & Simpson) for the intestinal bacterial diversity analysis of *M. nipponense* in two groups.

Diversity index		Feeding group	Starvation group
Richness estimators	Chao	2942.67 ± 187.70*	1969.33 ± 327.39
Diversity estimators	Shannon	8.24 ± 0.57	7.31 ± 0.46
	Simpson	0.98 ± 0.02	0.95 ± 0.02

Data are presented as mean ± SD ($n = 3$). Statistical significance is tested using an unpaired t -test ($*P < 0.05$).

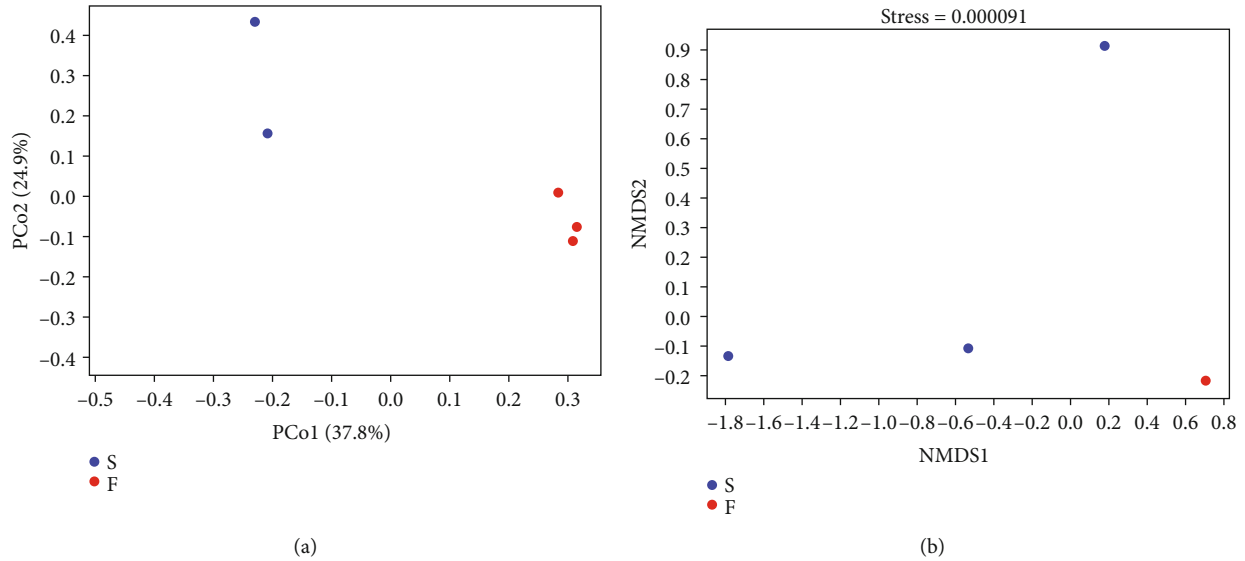


FIGURE 5: Beta diversity analysis of the gut microbiota of the F group and the S group of *M. nipponense* after 14 d of starvation: (a) principal coordinate analysis (PCoA); (b) nonmetric multidimensional scaling (NMDS) of Bray-Curtis distances. F: feeding group; S: starvation group.

measured by the disappearance rate of H_2O_2 at 240 nm [20]. One unit of CAT activity is defined as the amount of enzyme catalyzing the degradation of $1 \mu\text{mol}$ of $H_2O_2 \text{ min}^{-1}$ and the specific activity corresponding to μmol transformation of substrate (H_2O_2) $\text{min}^{-1} \text{mg}^{-1}$ protein. Lysozyme activity was measured with the turbidimetric method [21]. One unit of LZM activity was defined as the absorbance change in the cell suspension of *Micrococcus luteus* per min. Lyophilized *M. flavus* cells were resuspended in 2.0 mL of 0.05 M phosphate buffer (pH 6.2) at the concentration of 0.25 mg mL^{-1} and incubated at 30°C for 5 min, and then, $100 \mu\text{L}$ of the tissue supernatant was added to 2.0 mL of the suspension. The transmittance was measured after 5 s and 125 s at 540 nm. Lysozyme activity was defined as $(T_5 - T_{125}) / (S_5 - S_{125})$, where T was the sample transmittance and S was the standard transmittance.

2.4. Histological Assay. Gut segments were dehydrated with different gradients of alcohol concentration, cleaned in toluene, and embed in paraffin to make solid wax blocks. Then, the solid wax blocks were cut as continuous section blocks into sections $5 \mu\text{m}$ thick using a rotary microtome. The prepared paraffin sections were stained by hematoxylin and eosin (H&E) and observed with a light microscope (Nikon

Eclipse 80i, Tokyo, Japan). The epithelial cells and microvilli height of the gut were measured with a micrometer [22].

2.5. Gut DNA Extraction. The luminal contents of the gut were isolated, and the DNA of the total bacterial community were extracted using E.Z.N.A.™ Soil DNA Kit (Omega Bio-Tek, Norcross, USA) according to the manufacturer's instructions. The quality of extracted DNA samples was measured using a NanoDrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) and further confirmed by PCR amplification of the bacterial 16S rRNA gene.

2.6. Illumina High-Throughput Sequencing. The V3-V4 region of the 16S rRNA genes was amplified from the bacterial DNA template. The primers of 338F ($5'$ -ACTCCTACG GGAGGCAGCA- $3'$) and 806R ($5'$ -GGACTACHVGGGT WTCTAAT- $3'$) were used, which were modified with 7 bp barcodes to distinguish the PCR products. PCR amplification was performed according to the reported protocol of Shao et al. [23]. PCR products were detected by agarose gel electrophoresis and subsequently purified using an Axy-Prep™ DNA gel extraction kit (Axygen, Hangzhou, China). Each purified PCR product was sequenced with Illumina

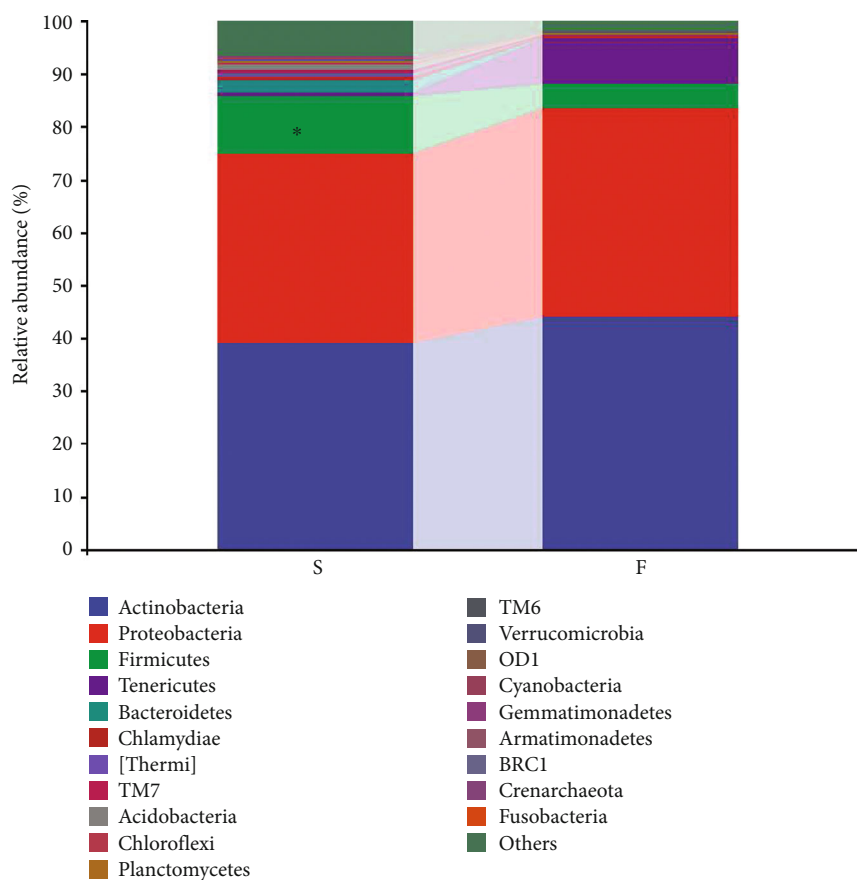


FIGURE 6: The abundance changes of the intestinal bacteria of *M. nipponense* at the phylum level after 14 d of starvation. Statistical significance is tested using an unpaired *t*-test (* $P < 0.05$).

high-throughput sequencing (Shanghai Personal Biotechnology Co., Ltd., Shanghai, China).

2.7. Processing of Illumina Sequencing Data. Raw FASTQ file reads were analyzed and quality-filtered. Operational taxonomic units (OTUs) were defined as sequences clustered with a threshold of 97% similarity using UCLUST [24]. Taxonomic richness estimators and community diversities were determined for each library in Mothur [25]. The species richness for each community was estimated based on OTU abundance matrices. Alpha diversity indices, Chao richness estimator, Shannon [26] and Simpson [27] diversity indexes, were used for assessing community diversity. Beta diversity indexes, including the principal coordinate analysis (PCoA) and nonmetric multidimensional scaling (NMDS), were performed to investigate the structural variation of microbial communities across samples using UniFrac distance metrics [28, 29]. The taxonomy compositions and abundances were visualized using GraphAn [30]. A Venn diagram was generated to represent the number of shared and unique species among groups and percentages (%) of OTUs. Normalized abundance was shown by heat map using pheatmap. Microbial functions were predicted with PICRUSt (version 1.1.0), based on high-quality sequences [31]. Predicted functional pathways were annotated by using the Kyoto Encyclopedia of Genes and Genomes (KEGG) at level 2 [32]. Redundancy

analysis (RDA) was performed using Canoco 5.0 to reveal the effect of digestive and immune enzymes on bacterial communities, and the significant factor was chosen according to P value ($P < 0.05$, [33]). To explore the interplay among shrimp digestion and immune enzyme activities, gut microbiota, and shrimp growth traits under starvation stress, the data matrix was fitted using structural equation modeling (SEM) based on the maximum-likelihood estimation method. The favorable model was confirmed using the nonsignificant χ^2 (chi-square) test ($P > 0.05$), root of mean square residual (RMR < 0.1), high goodness-of-fit index (GFI > 0.90), and root-mean-square error of approximation (RMSEA < 0.05). Only OTUs with IndVal values > 0.80 and significance < 0.05 were selected as indicators for distinguishing starved and feeding shrimp, as described in a previous study [34].

2.8. Data Analysis and Statistics. The data of enzyme activities were presented as the mean \pm standard deviation (SD) and were analyzed with an unpaired *t*-test. The value of $P < 0.05$ was set for the statistical significance level and $P < 0.01$ for very significance level.

2.9. Accession Number. The raw data in this study had been deposited in the NCBI Sequence Read Archive database. The accession number is PRJNA778555.

3. Result

3.1. Growth Performance, Digestive Enzyme Activity, and Intestinal Structure. After 14 d of starvation, the weight and the growth gain rate of the S group were significantly lower than those of the F group (Table 1). The activities of trypsin and lipase in the hepatopancreas of the S group were significantly lower after 4 d of starvation ($P < 0.05$). However, the activity of amylase in the hepatopancreas of the S group was significantly higher than that of the F group ($P < 0.05$) at 14 d after starvation (Figure 1). The intestinal epithelial cells of the F group were closely connected, and their microvilli were abundantly and neatly arranged on the free surface of the epithelial cells. On the contrary, intestinal epithelial cells of the S group were shrunken or even disappeared and the height of epithelial cells and microvilli simultaneously decreased (Figure 2). The height of epithelial cells and microvilli of the midgut from the S group shrimps at 14 d after starvation ($65.54 \pm 6.03 \mu\text{m}$) were significantly lower than those from the F group ($86.80 \pm 6.49 \mu\text{m}$) ($P < 0.05$).

3.2. Immune Enzyme Activity. The activities of immune-related enzymes, SOD, CAT, LZM, and ACP, in the hepatopancreas of the S group were consistently decreased after starvation. The activities of SOD, CAT, and ACP in the S group were significantly lower at 4 d after starvation when comparing with those in the F group ($P < 0.05$), and the decrease of LZM activity in the S group appeared at 7 d after starvation ($P < 0.05$) when comparing with those in the F group (Figure 3). However, the change of LZM activity to starvation stress was slow compared to the changes of the activities of SOD, CAT, and ACP.

3.3. Intestinal Microbiota Richness and Diversity Analysis. A total of 338,779 high-quality sequences were obtained from 6 samples of the intestinal bacteria, ranging from 50,795 to 62,676. The good coverage ranged from 0.989 to 0.997, which suggested that the complete microbial communities presented in the samples were identified completely. The sequences were clustered into operational taxonomic units (OTUs), and each OTU represented a unique phylotype. Finally, a total number of 14,285 OTUs were identified in all samples, and the number of OTUs detected in each sample ranged from 1569 to 3129. The total number of OTUs in the F group was significantly higher than that in the S group ($P < 0.05$) (Table 2), but 742 OTUs were shared by these two groups (Figure 4).

Bacterial richness and diversity were estimated with Chao, Shannon, and Simpson indices, respectively. The Chao estimator of the F group was significantly higher than that of the S group ($P < 0.05$), but the two groups showed no significant difference in the Shannon or Simpson index ($P > 0.05$, Table 3). The analysis of beta diversity by both PCoA and NMDS showed that samples of the F group were separated from those of the S group, indicating the potential influence of different feeding regimes on intestinal microbiota (Figure 5).

3.4. Bacterial Community Analysis and Comparison. In this study, total OTUs of bacterial community in all samples

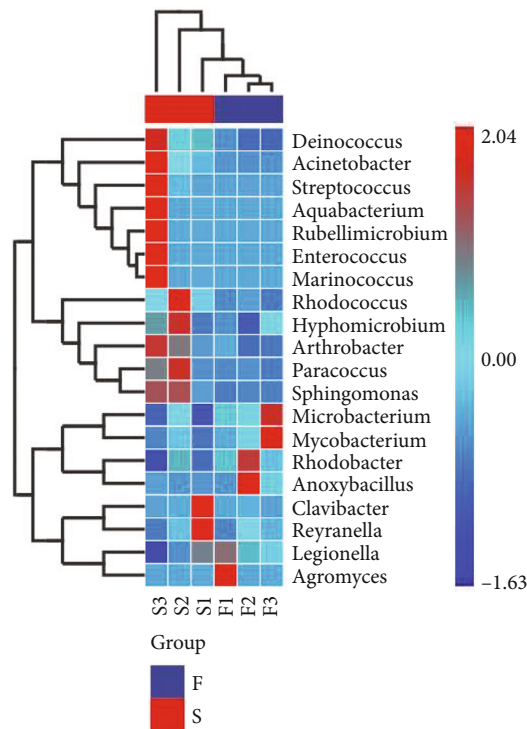


FIGURE 7: The abundance changes of the intestinal bacteria of *M. nipponense* at the genus level after 14 d of starvation by heat map. Phylogenetic positions are projected by the OTUs, and the taxa of OTUs are listed on the right. Color intensity indicates the relative enrichment of OTUs. F: feeding group (F1-F3); S: starvation group (S1-S3).

were classified into 30 phyla. The top 20 most abundant OTUs were shown in the taxonomic composition of intestinal microbiota at the phylum level (Figure 6), in which Actinobacteria and Proteobacteria were the predominant microflora phylum in the bacterial communities of both groups, and they accounted for $44.06 \pm 13.80\%$ and $39.44 \pm 11.21\%$ in the F group, respectively, and $39.06 \pm 12.00\%$ and $35.82 \pm 3.77\%$ in the S group, respectively. Firmicutes, Tenericutes, Bacteroidetes, and Chlamydiae were also abundant in all samples. The abundance of Bacteroidetes in the S group was significantly higher than that in the F group ($P < 0.05$).

The heat map of intestinal bacterial abundance in intestines at the genus level showed that the abundance of harmful bacteria and potential harmful bacteria, such as *Acinetobacter* [35], *Streptococcus* [36], *Enterococcus* [37], and *Rhodococcus* [38], in the S group were larger than those in the F group ($P < 0.05$), while the abundance of beneficial bacteria enriched in feeding prawns, such as *Microbacterium* [39], *Rhodobacter* ([40, 41]. [42]), and *Anoxybacillus* [43], were larger than in the S group ($P < 0.05$) (Figure 7). These results indicated that comparing to feeding prawns, starved ones had more opportunistic pathogenic bacteria but less beneficial bacteria in the intestine.

3.5. Functional Prediction of Intestinal Microbiota. To compare the functional potentials of the intestinal microbiota

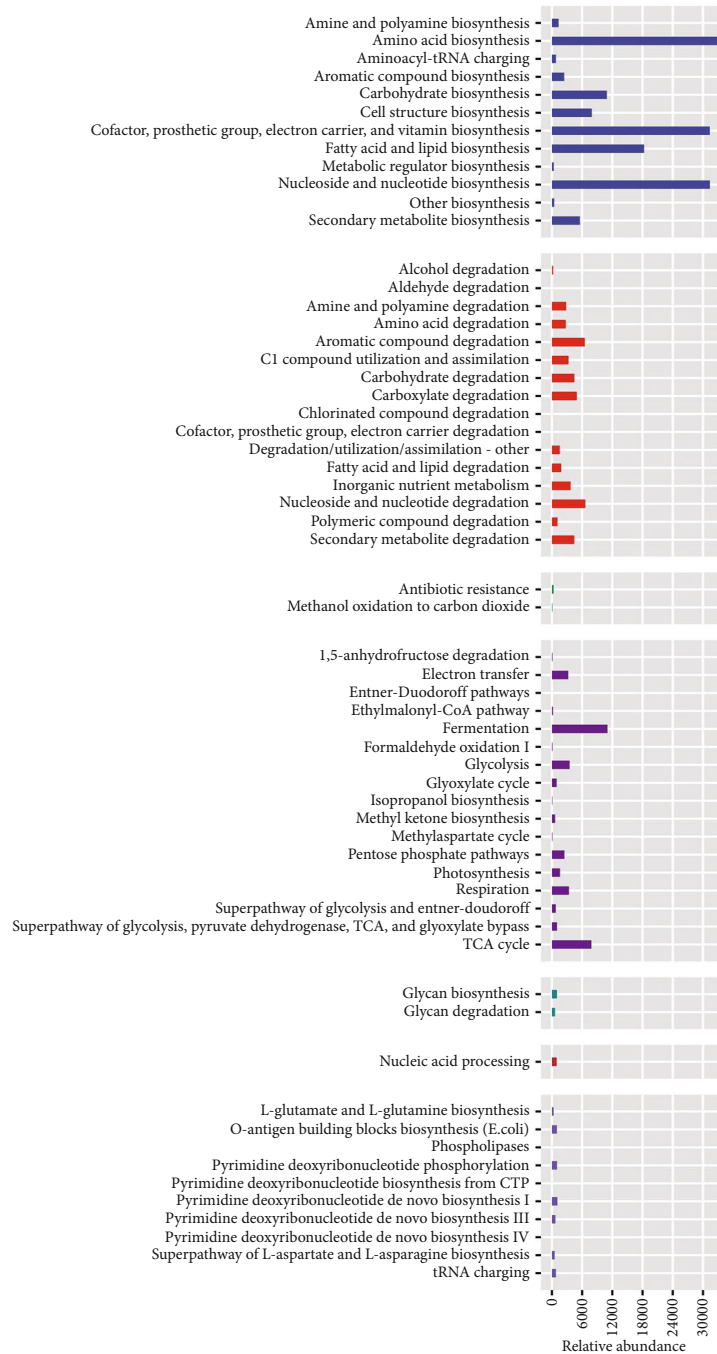


FIGURE 8: Relative abundance of functional pathways of the intestinal microbiota in *M. nipponense*. Pathways were predicted in the KEGG database at level 2.

between feeding and starved prawns, functional content was predicted in the KEGG database at level 2. Functional prediction showed that thirteen pathways including amino acid biosynthesis, cofactor, prosthetic group, electron carrier, vitamin biosynthesis, nucleoside and nucleotide biosynthesis, fatty acid and lipid biosynthesis, carbohydrate biosynthesis, cell structure biosynthesis, aromatic compound degradation, nucleoside and nucleotide degradation, fermentation, and TCA cycle were enriched in the intestinal microbiota after starvation (Figure 8). Meanwhile, the predicted nutrition metabolism of glycolysis V was significantly enriched in

the S group ($P < 0.05$). Moreover, the degradation of glucose, fucose, lactose, galactose, sucrose and rhamnose, L-arginine and L-ornithine degradation, peptidoglycan, and L-tryptophan biosynthesis were significantly decreased when the S group was compared with the F group ($P < 0.05$). This result implied that the gut microbiota of prawns adapted to the stress of starvation by decreasing nutrition metabolism to a certain degree. The immune system pathway of enterobacterial common antigen biosynthesis was markedly reduced in starved prawns ($P < 0.05$) (Figure 9). Nevertheless, for the terms of digestive-related metabolism (amino

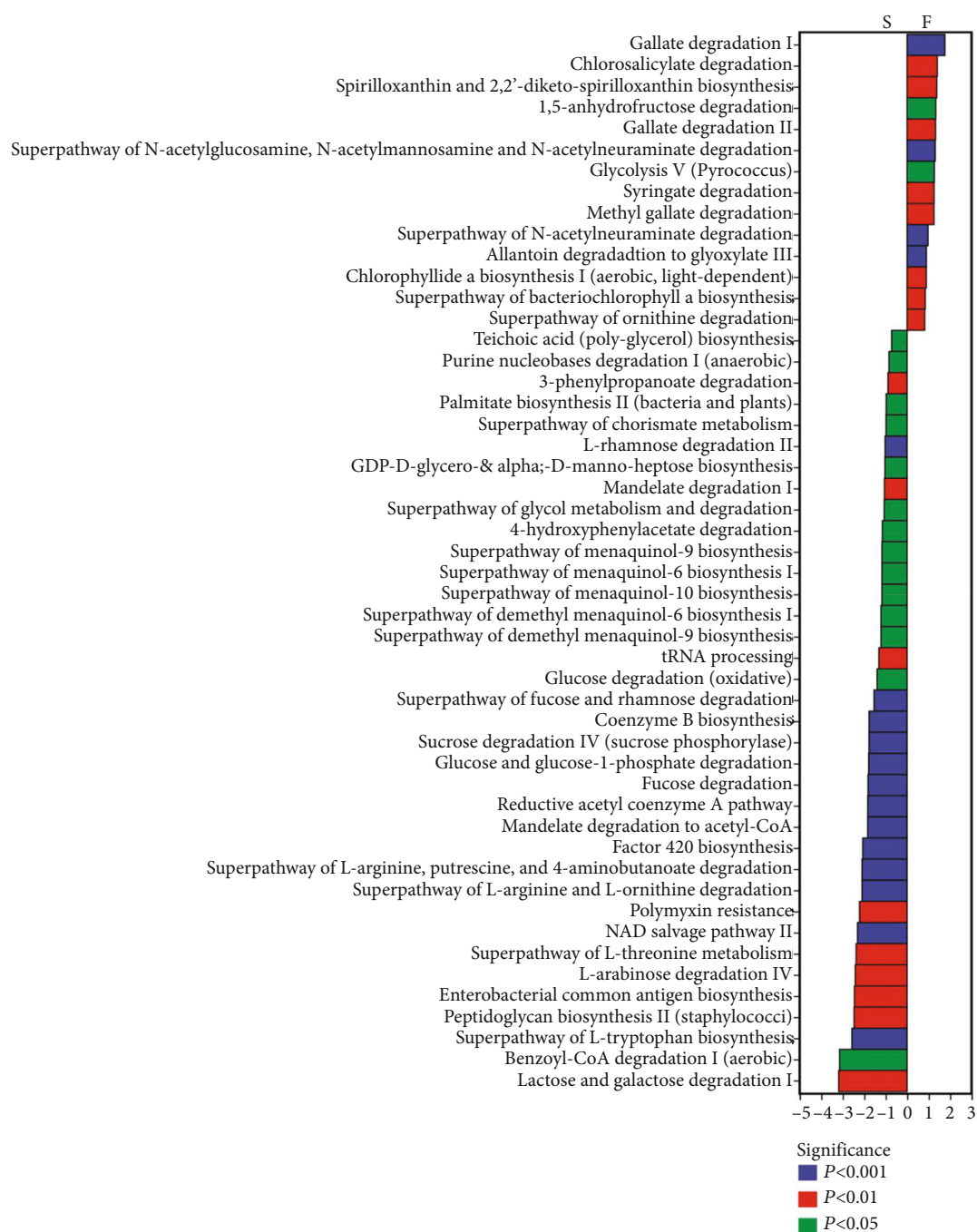


FIGURE 9: The changes of the KEGG pathways of intestinal bacteria in *M. nipponense* after 14 d of starvation. Pillars in the right column indicate significant increase, while those in the left column indicate significant decrease in the starvation group as compared with the feeding group in abundances of the corresponding pathways. F: feeding group; S: starvation group.

acid, carbohydrate, fatty acid, and lipid biosynthesis and degradation) and immune-related metabolism functions (antibiotic resistance, methanol oxidation to carbon dioxide, and phospholipases), there was no significant difference in the F and S groups ($P > 0.05$, Table 4).

3.6. The Interplay among Prawn Gut Bacterial Community, Digestive and Immune Enzyme Activity, and Growth. Redundancy analysis (RDA) was performed to examine

the relationships among microbial community structures, digestive enzyme activities, and immune enzyme activities (Figure 10(a)). The result showed that the composition of the intestinal bacterial flora in the S group had significant difference with that in the F group after 14 d of starvation. Meanwhile, the changes were potentially related to immune indicators SOD ($R^2 = 0.436$, P value = 0.188), LZM ($R^2 = 0.437$, P value = 0.175), ACP ($R^2 = 0.434$, P value = 0.207), and CAT ($R^2 = 0.424$, P value = 0.201) and digestive

TABLE 4: Relative abundance of digestive-related metabolism and immune-related metabolism functions in the starvation and feeding groups.

	Feeding group (%)	Starvation group (%)
Amino acid biosynthesis	14.73 ± 0.20	14.14 ± 0.32
Carbohydrate biosynthesis	4.76 ± 0.02	4.76 ± 0.32
Fatty acid and lipid biosynthesis	8.14 ± 0.82	7.96 ± 0.18
Amino acid degradation	1.20 ± 0.18	1.18 ± 0.25
Carbohydrate degradation	1.87 ± 0.23	1.95 ± 0.27
Fatty acid and lipid degradation	0.72 ± 0.03	0.79 ± 0.17
Antibiotic resistance	0.01 ± 0.09	0.21 ± 0.31
Methanol oxidation to carbon dioxide	0.03 ± 0.00	0.06 ± 0.03
Phospholipases	0.011 ± 0.01	0.01 ± 0.07

Note: there was no significant difference in two groups ($P > 0.05$).

indicators TRY ($R^2 = 0.424$, P value = 0.214), LPS ($R^2 = 0.459$, P value = 0.162), and AMS ($R^2 = 0.465$, P value = 0.166). In the RDA space, only AMS was negatively correlated with the microbial community structure in starved prawns, but the other indicators expressed positively with the microbial community structure. These indicators had an impact on the diversity and species composition of the bacterial flora of the S group.

The structural equation modeling (SEM) revealed the interplay among the gut bacterial community, digestive and immune enzyme activities, and weight gain rate (Figure 10(b)). The results indicated that changes of digestive and immune enzyme activities were directly related to the gut bacterial community and notably affected prawn growth.

4. Discussion

The stability and functional activities of gut microbiota are important to the host health. Gut microbiota enhanced the metabolic capabilities, modulated gastrointestinal development, balanced the immune response, and protected against pathogens for the host [44]. However, the relationship among starvation, digestion, immune activities, and intestinal microbiota community was rarely conducted in crustaceans. The studies showed that the change of gut bacterial community was directly related to digestive and immune enzyme activities under starvation stress in *L. vannamei* [8, 14]. In this present study, the difference of intestinal microbiota between starved and fed *M. nipponense* was studied using the method of high-throughput sequencing for the first time to elucidate the interplay among shrimp gut microbiota and digestive and immune enzyme activities after starvation.

The activity of digestive enzymes in aquaculture species was affected by growth, diet composition, natural feeding habits, and genetics [45, 46]. Therefore, digestive enzyme activities might reflect digestion physiology and nutritional changes under adverse environmental conditions [47]. In the present study, the digestive activities of trypsin and lipase in starved prawns were lower than those in normal

fed prawns. A few studies had shown that crustaceans could adapt to starvation stress by reducing the digestive activity in the shrimps *L. vannamei* [8, 48] and the crayfish *Procambarus clarkii* [46]. The changes of the digestive enzyme activities in shrimps reflected the availability of energy and nutrients; one possible reason for this phenomenon was that no food peristalsis mechanically stimulated the shrimp's entire digestive tracts, thereby reducing the secretion of digestive enzymes [49]. Alternatively, the depression of digestive activity could attenuate the utilization of energy reserves, thereby affording an adaptation strategy for animals to deal with food-depriving conditions and to prevent damage or death [4]. However, the contrary results were found in fish and shrimps as well. Researches had shown that the amylase activities in the hepatopancreas of common carp increased significantly after starvation [50], and trypsin and α -glucosidase activities in the hepatopancreas were significantly higher in starved shrimps than in fed ones under short-term starvation in *Penaeus monodon* [51]. Starvation triggered amino acid catabolism in digestive gland cells of the hepatopancreas and increased the specific activities of amylase, protease, trypsin, and chymotrypsin in *L. vannamei* [52]. In this present study, the activity of amylase exhibited an opposite trend with those of lipase and trypsin, which increased significantly after 14 d of starvation. This result agreed with previous studies performed on *M. nipponense* [5]. We suspected that it might be related with omnivorous feeding habits of *M. nipponense*. In addition, this view was further supported by the results of significant enrichment of glycolysis V in starved prawns (Figure 9). Maintenance of high levels of digestive enzyme activity even after the lack of feed might assure the utilization and absorption of the remaining food [50] and a relatively quick metabolic recovery when food becomes available [53].

The immune statuses of crustaceans were tightly linked with environmental factors [54, 55], diet supplementation [56, 57], and starvation [8]. The activities of SOD and CAT in *M. nipponense* decreased significantly with the rising of ROS under the stress of starvation [5] or sublethal concentration of nitrite [55]. In this study, the immune enzyme activities of SOD, CAT, LZM, and ACP in the hepatopancreas of starved prawns decreased and were consistently significantly lower than those of feeding ones (Table 4). The reduction of immune activities indicated that these enzymes might have been inhibited as a transitory response to food shortage [5]. By contrast, LZM, ACP, and SOD activities were consistently increased in starved *L. vannamei* [8], the CAT activity increased markedly in the hepatopancreas, and a contrary trend was observed in the hemolymph after white shrimp was exposed to ambient nitrite [54]. Reduction in the activities of digestive and immune enzymes under starvation might be an adaptation to feed scarcity and energy maintenance [52].

Dietary and food availability play a predominant role to shape the structure of animal gut microbial. Starvation is a major factor determining host gut microbiota [58]. In this study, the community structures of gut microbiota including the total number of OTUs and bacterial richness were significantly changed in the prawns after 14 d of starvation, while

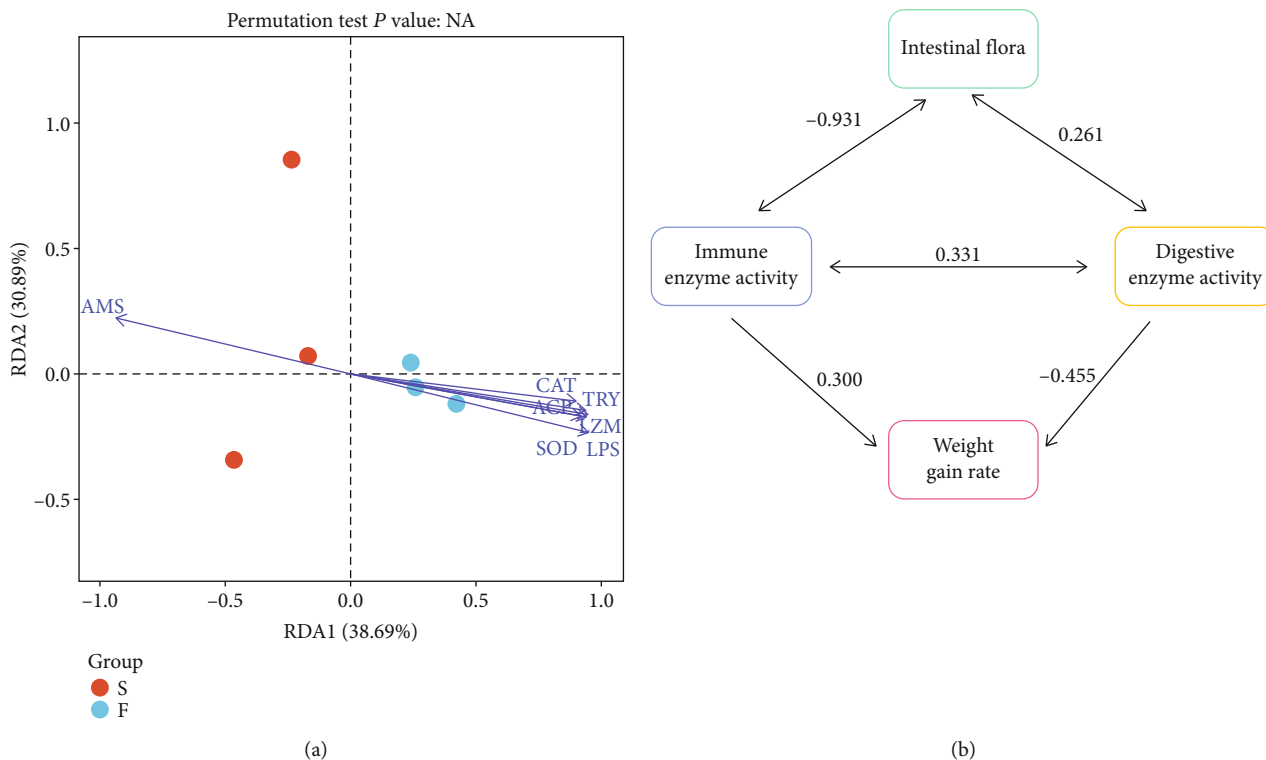


FIGURE 10: (a) The relationships between microbial community structures and digestive enzyme activity and the immune enzyme activity of the starvation group and the feeding group in *M. nipponense* with redundancy analysis (RDA). F: feeding group; S: starvation group. (b) The structural equation model (SEM) shows the interplay among the intestinal bacterial community, immune enzyme activity, digestive enzyme activity, and weight gain rate of *M. nipponense*. Numbers on the arrow are standardized paths calculated by Pearson correlation coefficient. Arrow widths show the strength of the causal relationship. R^2 of all paths (>0.8 , $P < 0.05$) indicated that the structural equation model analysis of variance results was statistically significant.

the Shannon and Simpson diversity indices were not significantly affected by starvation (Tables 2 and 3). In *L. vannamei*, the Shannon and Simpson indexes of gut microbiota were greatly changed under starvation stress [8]. Meanwhile, beta diversity in terms of PCoA and NMDS showed that the feeding group samples clustered separately from the starvation group samples, which indicated that feed deprivation limited the adaptive potential of the microbiota [58].

In the present study, the dominant microbiota phyla in *M. nipponense* gut were Actinobacteria and Proteobacteria, followed by Firmicutes, Tenericutes, Bacteroidetes, and Chlamydiae. The abundance of Actinobacteria and Proteobacteria was similar in the S group and the F group. It was not consistent with the results of previous studies conducted in *M. nipponense*, in which Proteobacteria was the most dominant bacteria in the intestine, followed by Firmicutes, Tenericutes, Bacteroidetes, Cyanobacteria, and Actinobacteria [59]. The result was also different in other shrimps such as *Macrobrachium rosenbergii* [60], *L. vannamei* [8, 23, 61], and black tiger shrimp [62]. Firmicute was the dominant phyla in humans and many land mammals [63–66]. In the present study, the relative abundance of Firmicutes in *M. nipponense* was relatively low, which were 4.41% in feeding prawns and 10.81% in starved ones. Similar result has also been found in *L. vannamei* [8, 23], which could be attributed to differences in phylogeny, living environment, and dietary

habits. Research showed that Proteobacteria and Firmicutes were the dominant microflora in the intestinal flora of healthy *Procambarus clarkii* [67] and were also used as indicators of fish health [68]. In this study, the abundance of Bacteroidetes in the S group was significantly higher than that in the F group. Moreover, the difference in dominant bacteria in starvation and feeding groups at the genus level was also found. There were more abundant harmful bacteria and potential harmful bacteria in starved prawns, while more beneficial bacteria were enriched in feeding prawns. The reduced immune system pathway, immune enzyme activity, and gut microbial diversity and increased quantities of potentially pathogenic bacteria indicated that starved prawns were more susceptible to pathogen infection.

Recently, many studies focused on the functional prediction of intestinal microbiota in shrimps. The severe changes of culture environmental conditions, diet composition, and health status could cause great difference on the metabolism functions of shrimp intestinal microbiota [8, 69–72]. When suffering the stress of starvation and white feces syndrome simultaneously, *L. vannamei* remarkably decreased the functional pathways involved in carbohydrate and protein digestion, glycan biosynthesis, and lipid and enzyme metabolism [8]. In the present study, the predicted nutrition metabolism involved in glycolysis V was significantly enriched after starvation. Meanwhile, partial carbohydrate and amino acid

metabolism and the pathways involved in immunity significantly decreased in starved prawns (Table 4). Nevertheless, the main nutrition-related metabolism functions including amino acid, carbohydrate, fatty acid, and lipid biosynthesis and degradation and immune-related metabolism functions including antibiotic resistance, methanol oxidation to carbon dioxide, and phospholipases were not significantly changed by starvation. Similarly, the dietary replacement did not reduce the significant difference in intestinal bacterial nutrition-related metabolism functions in *L. vannamei* [23]. These results implied that the gut microbiota of prawns could adapt to starvation by decreasing its metabolism level, but the short-term feed deprivation was insufficient to significantly change metabolism functions of intestinal microbiota in *M. nipponense*.

Gut microbiota had crucial effects on maintaining the mucosal structure, immunity, nutrition, and metabolism [12, 66]. Food deprivation or restriction was associated with alterations in the epithelial gut barrier [73]. In the present study, the significant decrease in the height of epithelial cells and microvilli in the S group suggested that starvation quickly damaged the structure of the prawn intestine, which might induce the change of the microbiota living environment. The increased abundance of Bacteroidetes, which possessed genes for a large number of enzymes involved in carbohydrate utilization [66, 74, 75], in starved prawns might be related to the increased activity of amylase, which was involved in glycolysis V metabolism. The results of redundancy analysis (RDA) showed a positive correlation between immune-related enzymes of SOD, CAT, LZM, and ACP; digestive enzymes of TRY and LPS, except AMS; and microbial community structure in starved prawns. The suppressed digestion and immune activities of starved prawns might be matched with the gut microbial richness and functions. It was supposed that energy homeostasis was kept under starvation condition by exploiting microbiota-derived sources of carbon and nitrogen as nutrients [76]. The result of SEM further revealed that there was a close correlation among prawn digestion and immune activities, gut microbiota, and growth traits. A similar result has also been found in *L. vannamei* [8].

5. Conclusions

In summary, the present study indicated that the changes of digestive and immune enzyme activities under the starvation condition were directly related to the gut bacterial community, which in turn affected the growth traits of prawns. Feed deprivation significantly changed the bacteria richness of the prawn gut microbiota. The increased abundance of Bacteroidetes in starved prawns might be related to the increased activity of amylase and glycolysis V metabolism. The starved prawns were more prone to be invaded by pathogens as evidenced by reduction of immune enzyme activity, the immune system pathway, and more abundant opportunistic pathogens. These results revealed the relationship among digestive enzymes, immune enzyme activities, and gut microbiota under starvation stress in *M. nipponense*.

Data Availability

The raw data in this study have been deposited in the NCBI Sequence Read Archive database which can be found online at <https://www.ncbi.nlm.nih.gov/sra/PRJNA778555>. The accession number is PRJNA778555.

Conflicts of Interest

The authors declare that they have no conflict of interest.

Authors' Contributions

GR and WS designed the experiments, analyzed the data, and wrote the manuscript. WS, YQ, HZ, and FM carried out the experiments. XZ wrote the manuscript. All authors carried out the revision of the manuscript and gave final approval for publication.

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References

- [1] K. B. Davis and T. G. Gaylord, "Effect of fasting on body composition and responses to stress in sunshine bass," *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology*, vol. 158, no. 1, pp. 30–36, 2011.
- [2] G. Caruso, M. G. Denaro, R. Caruso, F. Mancari, L. Genovese, and G. Maricchiolo, "Response to short term starvation of growth, haematological, biochemical and non-specific immune parameters in European sea bass (*Dicentrarchus labrax*) and blackspot sea bream (*Pagellus bogaraveo*)," *Marine Environmental Research*, vol. 72, no. 1–2, pp. 46–52, 2011.
- [3] C. A. Shoemaker, P. H. Klesius, C. Lim, and M. Yildirim, "Feed deprivation of channel catfish, *Ictalurus punctatus* (Rafinesque), influences organosomatic indices, chemical composition and susceptibility to *Flavobacterium columnare*," *Journal of Fish Diseases*, vol. 26, no. 9, pp. 553–561, 2003.
- [4] A. Sanchez-Paz, F. Garcia-Carreno, J. Hernandez-Lopez, A. Muhlia-Almazan, and G. Yepiz-Plascencia, "Effect of short-term starvation on hepatopancreas and plasma energy reserves of the Pacific white shrimp (*Litopenaeus vannamei*)," *Journal of Experimental Marine Biology and Ecology*, vol. 340, no. 2, pp. 184–193, 2007.
- [5] Z. H. Li, S. Xie, J. X. Wang, and D. Q. Chen, "Effects of intermittent starvation on growth and some digestive enzymes in the shrimp *Macrobrachium nipponense*," *Journal of Fisheries of China*, vol. 31, no. 4, pp. 456–462, 2007.
- [6] G. Ashouri, V. Yavari, M. Bahmani et al., "The effect of short-term starvation on some physiological and morphological parameters in juvenile Siberian sturgeon, *Acipenser baerii* (Actinopterygii: Acipenseriformes: Acipenseridae)," *Acta Ichthyologica et Piscatoria*, vol. 43, no. 2, pp. 144–149, 2013.
- [7] P. D. Zhang, X. M. Zhang, J. Li, and T. X. Gao, "Effect of refeeding on the growth and digestive enzyme activities of *Fenneropenaeus chinensis* juveniles exposed to different periods of food deprivation," *Aquaculture International*, vol. 18, no. 6, pp. 1191–1203, 2010.

- [8] W. F. Dai, J. J. Zhang, Q. F. Qiu et al., "Starvation stress affects the interplay among shrimp gut microbiota, digestion and immune activities," *Fish & Shellfish Immunology*, vol. 80, pp. 191–199, 2018.
- [9] S. Yengkokpam, D. Debnath, A. K. Pal et al., "Short-term periodic feed deprivation in *Labeo rohita* fingerlings: effect on the activities of digestive, metabolic and anti-oxidative enzymes," *Aquaculture*, vol. 412–413, pp. 186–192, 2013.
- [10] D. T. Hughes and V. Sperandio, "Inter-kingdom signalling: communication between bacteria and their hosts," *Nature Reviews Microbiology*, vol. 6, no. 2, pp. 111–120, 2008.
- [11] A. L. Kau, P. P. Ahern, N. W. Griffin, A. L. Goodman, and J. I. Gordon, "Human nutrition, the gut microbiome and the immune system," *Nature*, vol. 474, no. 7351, pp. 327–336, 2011.
- [12] A. O'Hara and F. Shanahan, "The gut flora as a forgotten organ," *EMBO Reports*, vol. 7, pp. 688–693, 2006.
- [13] D. L. Merrifield and E. Ringo, *Aquaculture Nutrition: Gut Health, Probiotics and Prebiotics*, John Wiley & Sons Press, Chichester, 2014.
- [14] W. Dai, W. Yu, J. Zhang, J. Zhu, Z. Tao, and J. Xiong, "The gut eukaryotic microbiota influences the growth performance among cohabitating shrimp," *Applied Microbiology and Biotechnology*, vol. 101, no. 16, pp. 6447–6457, 2017.
- [15] J. B. Feng, J. L. Li, and X. Cheng, "Research progress on germplasm resource exploitation and protection of *Macrobrachium nipponense*," *Journal of Shanghai Fisheries University*, vol. 17, pp. 371–376, 2008.
- [16] Z. H. Li, S. Xie, J. X. Wang, and D. Q. Chen, "The effects of starvation on growth and some immune function in the shrimp *Macrobrachium nipponense*," *Journal of Shanghai Fisheries University*, vol. 16, no. 1, p. 16–2, 2007.
- [17] M. M. Bradford, "A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding," *Analytical Biochemistry*, vol. 72, no. 1–2, pp. 248–254, 1976.
- [18] X. G. Liu, *Methods. The Guides of Biochemistry Experiment*, Higher Education Press, Beijing, 2006.
- [19] J. M. McCord and I. Fridovich, "Superoxide dismutase: an enzymic function for Erythrocyte protein (hemocuprein)," *The Journal of Biological Chemistry*, vol. 244, no. 22, pp. 6049–6055, 1969.
- [20] H. U. Bergmeyer, "Methods," in *Methods of Enzymatic Analysis*, D. H. Williamson, Ed., pp. 885–888, Academic Press, New York, 1963.
- [21] R. M. Parry, R. C. Chandan, and K. M. Shahani, "A rapid and sensitive assay of muramidase," *Proceedings of the Society for Experimental Biology and Medicine*, vol. 119, no. 2, pp. 384–386, 1965.
- [22] W. Du, H. Xu, X. Mei et al., "Probiotic *Bacillus* enhance the intestinal epithelial cell barrier and immune function of piglets," *Beneficial Microbes*, vol. 9, no. 5, pp. 743–754, 2018.
- [23] J. C. Shao, K. Y. Jiang, and L. Wang, "*Litopenaeus vannamei* fed diets with different replacement levels of fish meal by fish silage: a molecular approach on intestinal microbiota," *Aquaculture Nutrition*, vol. 25, no. 3, pp. 721–728, 2019.
- [24] R. C. Edgar, B. J. Haas, J. C. Clemente, C. Quince, and R. Knight, "UCHIME improves sensitivity and speed of chimera detection," *Bioinformatics*, vol. 27, no. 16, pp. 2194–2200, 2011.
- [25] P. D. Schloss, S. L. Westcott, T. Ryabin et al., "Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities," *Applied and Environmental Microbiology*, vol. 75, no. 23, pp. 7537–7541, 2009.
- [26] C. E. Shannon, "The mathematical theory of communication. 1963," *MD Computing*, vol. 14, no. 4, pp. 306–317, 1997.
- [27] W. F. Mahaffee and J. W. Kloepper, "Temporal changes in the bacterial communities of soil, rhizosphere, and endorhiza associated with field-grown cucumber (*Cucumis sativus* L.)," *Microbial Ecology*, vol. 34, no. 3, pp. 210–223, 1997.
- [28] C. A. Lozupone, M. Hamady, S. T. Kelley, and R. Knight, "Quantitative and qualitative β diversity measures lead to different insights into factors that structure microbial communities," *Applied and Environmental Microbiology*, vol. 73, no. 5, pp. 1576–1585, 2007.
- [29] C. A. Lozupone and R. Knight, "UniFrac: a new phylogenetic method for comparing microbial communities," *Applied and Environmental Microbiology*, vol. 71, no. 12, pp. 8228–8235, 2005.
- [30] F. Asnicar, G. Weingart, T. L. Tickle, C. Huttenhower, and N. Segata, "Compact graphical representation of phylogenetic data and metadata with GraPhlAn," *PeerJ*, vol. 3, article e1029, 2015.
- [31] M. G. I. Langille, J. Zaneveld, J. G. Caporaso et al., "Predictive functional profiling of microbial communities using 16S rRNA marker gene sequences," *Nature Biotechnology*, vol. 31, no. 9, pp. 814–821, 2013.
- [32] M. Kanehisa, S. Goto, Y. Sato, M. Furumichi, and M. Tanabe, "KEGG for integration and interpretation of large-scale molecular data sets," *Nucleic Acids Research*, vol. 40, pp. 109–114, 2012.
- [33] C. J. F. Ter Braak and P. Smilauer, *CANOCO Reference Manual and Cano Draw for Windows User's Guide: Software for Canonical Community Ordination (Version 4.5)*, Biometris, Wageningen, 2002.
- [34] J. B. Xiong, K. Wang, J. F. Wu et al., "Changes in intestinal bacterial communities are closely associated with shrimp disease severity," *Applied Microbiology and Biotechnology*, vol. 99, no. 16, pp. 6911–6919, 2015.
- [35] Z. L. Sun, Y. M. Xuan, H. Zhang et al., "Bacterial diversity of *Penaeus vannamei* Boone intestine and aquaculture environment," *Journal of Fisheries Science of Chinese*, vol. 23, no. 3, pp. 594–605, 2016.
- [36] Y. S. Lu, "Five years of reflection and examination on streptococcal disease of tilapia," *Ocean and Fisheries*, vol. 9, no. 59–59, p. 61, 2012.
- [37] J. H. Li, M. Y. Wang, X. L. Tian et al., "Effects of three lactic acid bacteria on growth, disease resistance and intestinal microflora structure of *Litopenaeus vannamei*," *Journal of Ocean University of China (Natural Science Edition)*, vol. 51, no. 4, pp. 44–54, 2021.
- [38] W. R. Finnerty, "THE BIOLOGY and GENETICS of the GENUS *RHODOCOCCUS*," *Annual Review of Microbiology*, vol. 46, no. 1, pp. 193–218, 1992.
- [39] L. Ming, X. L. Dai, and Z. B. Jiang, "Application of high-throughput sequencing in the comparison of disease resistance of *Macrobrachium rosenbergii*," *Journal of Henan Agricultural Sciences*, vol. 47, no. 8, pp. 134–141, 2018.
- [40] X. X. Dong, L. L. Lv, W. H. Zao, Y. B. Yu, and Q. G. Liu, "Study on the structure of intestinal microflora of *Macrobrachium*

- rosenbergii* under different culture patterns and its relationship with environmental factors,” *Journal of Shanghai Ocean University*, vol. 28, no. 4, pp. 501–510, 2019.
- [41] S. Y. Zhang, M. Q. Xie, L. L. Xu et al., “Effects of chitosan oligosaccharide on digestive enzyme activities, intestinal microflora structure, serum non-specific immune indexes and disease resistance of *Procambarus clarkii*,” *Chinese Journal of Animal Nutrition*, vol. 32, no. 12, pp. 5864–5874, 2020.
- [42] J. L. Balcázar, I. De Blas, I. Ruiz-Zarzuola, D. Cunningham, D. Vendrell, and J. L. Múzquiz, “The role of probiotics in aquaculture,” *Veterinary Microbiology*, vol. 114, no. 3-4, pp. 173–186, 2006.
- [43] M. Y. Wang, X. L. Tian, L. Z. Liu et al., “Effects of *Bacillus coagulans* supplementation on growth, serum nonspecific immune indices and disease resistance of *Litopenaeus vannamei*,” *Journal of Ocean University of China*, vol. S1, pp. 8–15, 2018.
- [44] C. Almansa, A. Agrawal, and L. A. Houghton, “Intestinal microbiota, pathophysiology and translation to probiotic use in patients with irritable bowel syndrome,” *Expert Review of Gastroenterology & Hepatology*, vol. 6, no. 3, pp. 383–398, 2012.
- [45] L. Charron, O. Geffard, A. Chaumot et al., “Consequences of lower food intake on the digestive enzymes activities, the energy reserves and the reproductive outcome in *Gammarus fossarum*,” *PLoS One*, vol. 10, no. 4, article e0125154, 2015.
- [46] H. J. Sacristán, M. Ansaldo, L. M. Franco-Tadic, A. V. Fernández Gimenez, and L. S. López Greco, “Long-term starvation and posterior feeding effects on biochemical and physiological responses of midgut gland of *Cherax quadricarinatus* juveniles (Parastacidae),” *PLoS One*, vol. 11, no. 3, article e0150854, 2016.
- [47] J. Sunde, S. A. Eiane, A. Rustad et al., “Effect of fish feed processing conditions on digestive protease activities, free amino acid pools, feed conversion efficiency and growth in Atlantic salmon (*Salmo salar* L.),” *Aquaculture Nutrition*, vol. 10, no. 4, pp. 261–277, 2004.
- [48] C. Pascual, A. Sanchez, E. Zenteno et al., “Biochemical, physiological, and immunological changes during starvation in juveniles of *Litopenaeus vannamei*,” *Aquaculture*, vol. 251, no. 2-4, pp. 416–429, 2006.
- [49] A. Muhlia-Almazán and F. L. García-Carreño, “Influence of molting and starvation on the synthesis of proteolytic enzymes in the midgut gland of the white shrimp *Penaeus vannamei*,” *Comparative Biochemistry and Physiology Part B: Biochemistry & Molecular Biology*, vol. 133, no. 3, pp. 383–394, 2002.
- [50] Y. N. Wang, Z. R. Zhang, and S. M. Zheng, “Compensatory growth and effects of starvation on amylase in common carp,” *Freshwater Fisheries*, vol. 21, no. 5, pp. 6-7, 2001.
- [51] B. L. Ong and D. Johnston, “Influence of feeding on hepatopancreas structure and digestive enzyme activities in *Penaeus monodon*,” *Journal of Shellfish Research*, vol. 25, no. 1, pp. 113–121, 2006.
- [52] L. Comoglio, G. Gaxiola, A. Roque, G. Cuzon, and O. Amin, “The effect of starvation on refeeding, digestive enzyme activity, oxygen consumption, and ammonia excretion in juvenile white shrimp *Litopenaeus vannamei*,” *Journal of Shellfish Research*, vol. 23, no. 1, pp. 243–249, 2004.
- [53] M. Furné, M. García-Gallego, M. C. Hidalgo Carmen et al., “Effect of starvation and refeeding on digestive enzyme activities in sturgeon (*Acipenser naccarii*) and trout (*Oncorhynchus mykiss*),” *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology*, vol. 149, no. 4, pp. 420–425, 2008.
- [54] S. Liao, Q. Li, A. Wang et al., “Effect of nitrite on immunity of the white shrimp *Litopenaeus vannamei* at low temperature and low salinity,” *Ecotoxicology*, vol. 21, no. 6, pp. 1603–1608, 2012.
- [55] W. N. Wang, A. L. Wang, Y. J. Zhang et al., “Effects of nitrite on lethal and immune response of *Macrobrachium nipponense*,” *Aquaculture*, vol. 232, no. 1-4, pp. 679–686, 2004.
- [56] Y. H. Chien, C. H. Pan, and H. Hunter, “The resistance to physical stresses by *Penaeus monodon* juveniles fed diets supplemented with astaxanthin,” *Aquaculture*, vol. 216, no. 1-4, pp. 177–191, 2003.
- [57] W.-Y. Shen, L.-L. Fu, W.-F. Li, and Y.-R. Zhu, “Effect of dietary supplementation with *Bacillus subtilis* on the growth, performance, immune response and antioxidant activities of the shrimp (*Litopenaeus vannamei*),” *Aquaculture Research*, vol. 41, no. 11, pp. 1691–1698, 2010.
- [58] C. De Filippo, D. Cavalieri, M. Di Paola et al., “Impact of diet in shaping gut microbiota revealed by a comparative study in children from Europe and rural Africa,” *Proceedings of the National Academy of Sciences*, vol. 107, no. 33, pp. 14691–14696, 2010.
- [59] Y. F. Xiong, J. W. Wu, D. S. Zhou et al., “Effects of yeast hydrolyzate on growth, antioxidant, immune and intestinal flora of *Macrobrachium nipponense*,” *Chinese Journal of Animal Nutrition*, vol. 33, no. 4, pp. 2166–2212, 2021.
- [60] S. Miao, J. Zhu, C. Zhao, L. Sun, X. Zhang, and G. Chen, “Effects of C/N ratio control combined with probiotics on the immune response, disease resistance, intestinal microbiota and morphology of giant freshwater prawn (*Macrobrachium rosenbergii*),” *Aquaculture*, vol. 476, pp. 125–133, 2017.
- [61] M. L. Zhang, Y. H. Sun, K. Chen et al., “Characterization of the intestinal microbiota in Pacific white shrimp, *Litopenaeus vannamei*, fed diets with different lipid sources,” *Aquaculture*, vol. 434, pp. 449–455, 2014.
- [62] W. Rungrassamee, A. Klanchui, S. Maibunkaew, S. Chaiyapechara, P. Jiravanichpaisal, and N. Karoonuthaisiri, “Characterization of intestinal bacteria in wild and domesticated adult black tiger shrimp (*Penaeus monodon*),” *PLoS One*, vol. 9, no. 3, article e91853, 2014.
- [63] M. Dehingia, K. T. Devi, N. C. Talukdar et al., “Gut bacterial diversity of the tribes of India and comparison with the worldwide data,” *Scientific Reports*, vol. 5, no. 1, article 18563, 2015.
- [64] P. Fan, P. Liu, P. Song, X. Chen, and X. Ma, “Moderate dietary protein restriction alters the composition of gut microbiota and improves ileal barrier function in adult pig model,” *Scientific Reports*, vol. 7, no. 1, article 43412, 2017.
- [65] J. F. Garcia-Mazcorro, I. Ivanov, D. A. Mills, and G. Noratto, “Influence of whole-wheat consumption on fecal microbial community structure of obese diabetic mice,” *PeerJ*, vol. 4, p. e1702, 2016.
- [66] B. S. Ramakrishna, “Role of the gut microbiota in human nutrition and metabolism,” *Journal of Gastroenterology and Hepatology*, vol. 28, pp. 9–17, 2013.
- [67] Y. M. Chen, Z. M. Gu, L. J. Li, T. S. Chen, and J. F. Yuan, “Effect of white spot syndrome virus (WSSV) infection on intestinal flora composition of *Procambarus clarkia*,” *Journal of Huazhong Agricultural University*, vol. 39, no. 2, pp. 40–46, 2020.

- [68] M. J. Foysal, M. Alam, F. Momtaz et al., "Dietary supplementation of garlic (*Allium sativum*) modulates gut microbiota and health status of tilapia (*Oreochromis niloticus*) against-Streptococcus iniaeinfection," *Aquaculture Research*, vol. 50, no. 8, pp. 2107–2116, 2019.
- [69] F. Cornejo-Granados, A. A. Lopez-Zavala, L. Gallardo-Becerra et al., "Microbiome of Pacific whiteleg shrimp reveals differential bacterial community composition between wild, aquacultured and AHPND/EMS outbreak conditions," *Scientific Reports*, vol. 7, no. 1, p. 11783, 2017.
- [70] D. Hou, Z. Huang, S. Zeng et al., "Intestinal bacterial signatures of white feces syndrome in shrimp," *Applied Microbiology and Biotechnology*, vol. 102, no. 8, pp. 3701–3709, 2018.
- [71] S. Zeng, Z. Huang, D. Hou, J. Liu, S. Weng, and J. He, "Composition, diversity and function of intestinal microbiota in pacific white shrimp (*Litopenaeus vannamei*) at different culture stages," *PeerJ*, vol. 5, article e3986, 2017.
- [72] M. Zhang, Y. Sun, Y. Liu et al., "Response of gut microbiota to salinity change in two euryhaline aquatic animals with reverse salinity preference," *Aquaculture*, vol. 454, pp. 72–80, 2016.
- [73] L. Genton, P. D. Cani, and J. Schrenzel, "Alterations of gut barrier and gut microbiota in food restriction, food deprivation and protein-energy wasting," *Clinical Nutrition*, vol. 34, no. 3, pp. 341–349, 2015.
- [74] H. J. Flint, K. P. Scott, S. H. Duncan, P. Louis, and E. Forano, "Microbial degradation of complex carbohydrates in the gut," *Gut Microbes*, vol. 3, no. 4, pp. 289–306, 2012.
- [75] M. Zhang, J. R. Chekan, D. Dodd et al., "Xylan utilization in human gut commensal bacteria is orchestrated by unique modular organization of polysaccharide-degrading enzymes," *PNAS*, vol. 111, no. 35, pp. E3708–E3717, 2014.
- [76] M. D. McCue, "Starvation physiology: reviewing the different strategies animals use to survive a common challenge," *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology*, vol. 156, no. 1, pp. 1–18, 2010.