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Research Article 研究报告

Growth variance and intestinal microbial diversity of *Litopenaeus vannamei* raised in high-nitrite environment

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Abstract: [Objective] Pacific white shrimp (*Litopenaeus vannamei*) raised in high-nitrite environment tend to have different growth rates and body weights between individuals at the end of the culture period. This study aims to investigate the association between growth variance with intestinal microbiota structure of shrimps raised in a high-nitrite environment. **[Methods]** Intestinal contents and seawater samples were collected from rapidly growing, normally growing, and slowly growing shrimps raised in a high-nitrite pond for 16S rRNA gene sequencing and linear discriminant analysis effect size (LEfSe). **[Results]** The intestinal microbial diversity of the slowly growing shrimps was different from that of rapidly and normally growing shrimps. The principal coordinate analysis showed that the intestinal microbiota structure of the normally growing shrimps was more similar to that of the rapidly growing shrimps than to that of the slowly growing shrimps. The results of LEfSe revealed that *Flammeovirgaceae*, *Flavobacteriaceae*, and *Planctomycetaceae* had high abundance in the shrimps with rapid growth, while *Desulfovibrionaceae*, *Shewanellaceae*, and *Vibrionaceae* were significantly enriched in the shrimps with slow growth. **[Conclusion]** The difference in nitrogen metabolism capacity of intestinal microorganisms leads to the growth variance among shrimps in the high-nitrite environment. The findings provide new knowledge for the industrial farming of shrimps.

Keywords: *Litopenaeus vannamei*; high nitrite; 16S rRNA; intestinal microbiota

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高亚硝酸盐环境下不同生长速率凡纳滨对虾肠道微生物多样性研究

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摘　要：【目的】高亚硝酸盐环境中饲养的凡纳滨对虾(*Litopenaeus vannamei*)，在养殖结束时其生长速率和体重往往差异较大。本研究旨在探讨在高亚硝酸盐环境下饲养的对虾生长速率与肠道菌群结构和功能的相关性。【方法】本研究通过收集高亚硝酸盐条件下快速生长对虾(rapidly growing, RG)、正常生长对虾(normally growing, NG)和缓慢生长对虾(slowly growing, SG)的肠道和海水样品，通过 16S rRNA 基因测序、线性判别分析[line discriminant analysis (LDA) effect size, LEfSe]等进行分析。【结果】发现 SG 的细菌群落多样性与 RG 和 NG 不同。主坐标分析(principal coordinate analysis, PCoA)分析表明，NG 的群落组成与 RG 比 SG 更相似。通过 LEfSe 差异分析发现，RG 中火色杆菌科(*Flammeovirgaceae*)、黄杆菌科(*Flavobacteraceae*)和浮酶菌科(*Planctomycetaceae*)的丰度较高，而 SG 中脱硫弧菌科(*Desulfovibrionaceae*)、希瓦氏菌科(*Shewanellaceae*)和弧菌科(*Vibrionaceae*)的丰度显著增加。【结论】本研究发现，在高亚硝酸盐环境下，肠道微生物群落的氮代谢能力是造成对虾不同生长速度的原因。该研究将为虾的工业化养殖提供指导。

关键词：凡纳滨对虾；高亚硝酸盐；16S rRNA；肠道微生物

Litopenaeus vannamei, native to the Pacific coast of Central and South America, is the most important species of cultured shrimp in the world because of its strong adaptability to the environment and high economic value. After its introduction to China, the scale of cultivation has been continuously increased, which plays an important role in promoting local economic development. After its development for decades, earthen pond culture, the common and traditional farming model, has faced challenges such as high use of antimicrobials, safety hazards, and environmental pollution. Zero-water-exchanging industrial shrimp culture emerges due to the advancement of science and technology. This new farming technology has many advantages such as limited impact from climatic environment, water saving, land saving, environmental protection, high productivity, and seafood quality and safety. Although the modernization of aquaculture has been moving in

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the direction of zero-water- exchanging model, this farming model also has some problems to solves[1-2]. Among those problems, high nitrite concentration in the water during the late stage of farming is the most prominent one[3]. Nitrite is an intermediate in the conversion of ammonia to nitrate. Nitrite concentration in the water gradually increases as the day of culture increases. The nitrite ion enters the blood circulation through the respiratory system of the shrimp, and inhibits the oxygen carrying capacity of the blood, resulting in hypoxia or poisoning of the cultured aquatic animal[4] and causing the slow growth of shrimp. And recently, shrimps of different sizes in the same pool of high nitrite were found in this work.

Now it has been recognized that the gut microbiome acts as a virtual endocrine organ by acting as a barrier against pathogen invasion and providing complementary metabolic pathways for host nutrient access[5-6]. Intestinal is the best habitat for vast microbial communities in animal hosts which contribute to many crucial functions such as digestion. Previous researchers have shown that the microbiota in the digestive tract contributes to the digestion and absorption of nutrients as well as specific growth rates, immunity and antagonism of host pathogens[7-9]. There is increasing evidence that developmental stages[10], diet[11], health status[12], and feeding environmental conditions[13] are the main factors determining the composition and function of the intestinal community. A recent study showed that sea cucumbers under the same culture environment had different growth rates and that their intestinal bacterial composition was clearly different at the species level[14]. Similarly, it was observed that the body size and weight were significantly different of shrimp raised in a high-nitrite pool. A generally accepted explanation is that variations in intestinal microflora between individuals lead to different growth rates[5-6]. But the differences of gut microbes in the same managed shrimp cannot be attributed to the above drivers. From both commercial and scientific perspectives, it is important to understand the relationship between shrimp gut community assembly and growth rate of shrimp raised in high nitrite environment.

Previous studies on the microbial diversity of shrimp culture environment were limited by the technology itself and therefore it is difficult to analyze the bacterial community composition comprehensively and deeply in those studies. The development of high-throughput sequencing technology based on 16S rRNA gene fragment has gradually lifted the limitation and made it feasible to more comprehensively analyze the types and quantities of bacteria in microbial communities[15]. This study revealed the composition and dynamics of intestinal bacterial communities of *L. vannamei* cultured in the high nitrite circumstance over the farming period.

1 Materials and Methods

1.1 Experimental design and sample collection

The shrimp were raised in a concrete pond (length×width×height=2.8 m×3.8 m×0.8 m) at the Aquatic Experiment Base of Guangzhou, Guangdong Province in South China (23°06′N, 113°15′E, and 11 m in elevation). The pond was washed three times with fresh water, disinfected, then filled with diluted seawater which had the salinity 5‰ and the concentration of nitrite less than 0.01 mg/L). The aeration was carried out throughout the day. On March 22, 2018, 2 000 shrimp larvae (0.8–1.0 cm) were stocked in the pond and fed daily at 5%–10% of body weight. The nitrite concentration in water and body length and weight of shrimps were measured regularly. On May 18, the nitrite concentration in the water had sharp increase by more than 0.4 mg/L on May 18 and continued to increase until it reached 4 mg/L on May 25. Afterwards, it remained at relatively stable level, which (measured once a week, NMDCX0000175, Table S1) was much higher than normal level (less than 0.2 mg/L), until June 20, when the 90-day culture ended. The main water quality parameters were: nitrite 4 mg/L,

ammonia nitrogen 0.2 mg/L, total hardness 16.87 mmol/L, pH 7.6–7.8, dissolved oxygen 6.46 mg/L. At the end of the culture, shrimp were classified as three groups according to their body weight: the rapidly growth group (RG) (top 3% biggest body weight shrimp); the normally growth group (NG) (50% medium body weight shrimp); the slow growth group (SG) (the bottom 3% smallest body weight shrimp). Six shrimps of each group were collected and dissected on ice. Their intestines were combined to form a sample with three replicates per sample. One hundred mL water was filtered with a 0.22 μm pore size sterile membrane (millipore), then packaged in EP tube, frozen in a –80 °C ultra-low temperature freezer.

1.2 DNA extraction and 16S rRNA gene sequencing

Genomic DNA was extracted from the samples using bacterial genomic DNA QIAamp DNA Stool Mini Kit (QIAGEN) according to the manufacturer's instructions. After extracting genomic DNA from the sample, the V3+V4 region of 16S rRNA gene was amplified using a specific primer with a barcode. The primer sequences were: 341F: 5′-CCTACGGGNGGCWGCAG-3′; 806R: 5′-GGACTACHVGGGTATCTAAT-3′. The PCR amplified product was then recovered and quantified using a QuantiFluor™ fluorometer. The purified amplification products were mixed in equal amounts, and the sequencing adaptors were ligated to construct a sequencing library, and the HiSeq 2500 PE250 was sequenced on the machine. All the raw data were available on NCBI (No. PRJNA685743).

1.3 Bioinformatics analysis

The FLASH software was used to filter the raw data according to the barcode and primer sequence under the following rules: (1) Removal of sequences with an excessive N ratio: removal of sequences with more than 10% N bases in reads; (2) Removal of the reads which bases with a quality value higher than 20 and the number of bases was less than 40% of the total number of bases; (3) According to the overlapping relationship between PE reads, the paired double-ended reads were spliced into one sequence Raw Tags (the splicing condition is that the minimum matching length is 10 bp, and the overlapping area allowed the mismatch rate is 2%); (4) high quality Tags data (clean tags) were obtained according to Qimes (v1.9.1) Tags quality control process[16]; (5) The tags sequence obtained after the above processing were compared with a database (Gold database r20 110 519), and the chimeric sequences were removed to obtain the final effective data (effective tags)[17]. The tag sequence was de-duplicated using the Mothur (v1.39.1) software package, and the unique tag sequence was selected. The all-effective tags sequence of all samples was clustered using Uparse (usearch v9.2.64) software. By default, the sequence was clustered into OTUs (operational taxonomic units) with 97% identity (identity), and the absolute abundance and relative information of each OTU in each sample were calculated. The process of constructing OTUs will be representative. The RDP Classifier (version 2.2) was used to perform species annotations with the species-annotated database (set the confidence threshold to 0.8–1.0). The QIIME software was used to calculate the alpha diversity of each sample. The Venn diagram was drawn in the R language Venn Diagram (v1.6.17), and the OTUs contained in each set were counted. Combined with the abundance information of the OTU, the G UniFrac (v1.0) package in the R language was used to calculate the two samples. Distance between unweighted Unifrac and weighted Unifrac. Finally, multivariate statistical method principal co-ordinates analysis (PCoA), non-metric multidimensional scaling (NMDS), unweighted pair-group method with arithmetic means. Using FUNGuild, Tax4fun, FAPROTAX, BugBase, and other forecasting software were used to perform targeted prediction of community function according to different data types. The specific major flora between the groups by LEfSe (line discriminant analysis (LDA) effect size, LEfSe) (v1.0) analysis of the differences between groups of bacteria.

1.4 Statistical analysis

All statistical analyses were performed with SPSS (Windows version 20.0) and considered $P<0.05$ to be significant for all statistical tests. The bacterial abundance in different groups were analyzed by one-way ANOVA. All results were shown as mean±SD. Non-normally distributed data were converted to natural logarithms. Differences groups were analyzed with one-way ANOVA followed by LSD post hoc comparisons.

2 Results and Analysis

2.1 Shrimp categories and growth

The shrimp performance was negatively impacted over time decreased during the culture period (Table 1). The average body length of RG, NG, and SG groups was (10.68±0.32) cm, (9.45±0.12) cm, and (7.15±0.30) cm, respectively. The average body weight of RG, NG, and SG groups was (8.21±0.61) g, (5.49±0.27) g and (2.36±0.08) g, respectively. Significant differences were found among the three groups (body weight: ANOVA, $F_{(2,15)}$=278.7, P=0.000; length: ANOVA, $F_{(2,15)}$=335.8, P=0.000; Table 2).

2.2 Distribution of taxa and phylotypes

A total of 773 505 V3–V4 denoising 16S rRNA gene readings were obtained, with an average of 77 351 raw PE readings per sample (the minimum is 58 985 in one sample and the maximum is 111 747, NMDCX0000175, Table S2). All effective tags sequences of all samples were clustered using Uparse (usearch v9.2.64) software. By default, sequences were clustered into OTUs with 97% consistency (NMDCX0000175, Table S3), and the valid average OTUs reads was 526. The dilution curve tended to approach the saturation platform, indicating that the 16S rRNA gene sequence database depth was sufficient for microbial diversity analysis.

Table 1 Shrimp performance over time during the culture period

Date	IW (g)	FW (g)	SR (%)	WGR (%)	SGR (%)
5/30	3.37±0.14	3.93±0.08	100	16.65±3.41	2.19±0.42
6/6	3.90±0.07	4.73±0.03	100	21.29±1.35	2.76±0.16
6/13	4.79±0.01	5.17±0.05	97	7.93±0.72	1.09±0.10
6/19	5.15±0.07	5.46±0.07	95	6.16±0.23	0.10±0.04

The date represents the year 2018. Data are means±SE (n=50). IW (g): Initial weight; FW (g): Final weight; SR (%): Survival rate=number of surviving/total number×100%; WGR (%): Weight gain rate=(FW−IW)/IW; SGR (%): Specific growth rate=(FW−IW)/culturing day.

Table 2 Body length and body weight of three groups of shrimp

Groups	Body length (cm)	Body weight (g)
RG	10.68±0.32^a	8.21±0.61^a
NG	9.45±0.12^b	5.49±0.27^b
SG	7.15±0.30^c	2.36±0.08^c
P	0.000	0.000

Different superscripts in each row mean difference within groups. Each value is the mean±SD. Different letters (a, b, c) represent a significant difference within groups (one-way ANOVA).

After the effective tags were acquired, all sequences were classified from phylum to genus based on the RDP classifier[18]. Twenty-seven different phyla were detected in all samples, and the top 10 phyla with an abundance of 2% in at least one sample were shown in the Figure 1. *Proteobacteria* was the major microflora of all samples, followed by *Actinobacteria*, *Bacteroidetes*, *Chloroflexi*, *Verrucomicrobia*, *Planctomycetes*, *Firmicutes* (NMDCX0000175, Figure S1). At the phylum level, the abundance of *Proteobacteria* in the SG group was significantly higher than that in the other groups. At the genus level, *Photobacterium* and *Vibro* in the SG group were significantly higher than those in the other groups. Compared to SG group, *Haloferula* was higher in the RG and NG groups, indicating that the shrimp host exerted selective pressure on the bacterial population. In order to clarify the common and unique information of OTUs between different groups, the Venn Diagram (v1.6.17) package of the R language was used to map the Venn graphs, and the OTUs contained in each set were counted (Figure 2). To compare the differences between the groups better, 214 species shared by each group were selected out for subsequent analysis.

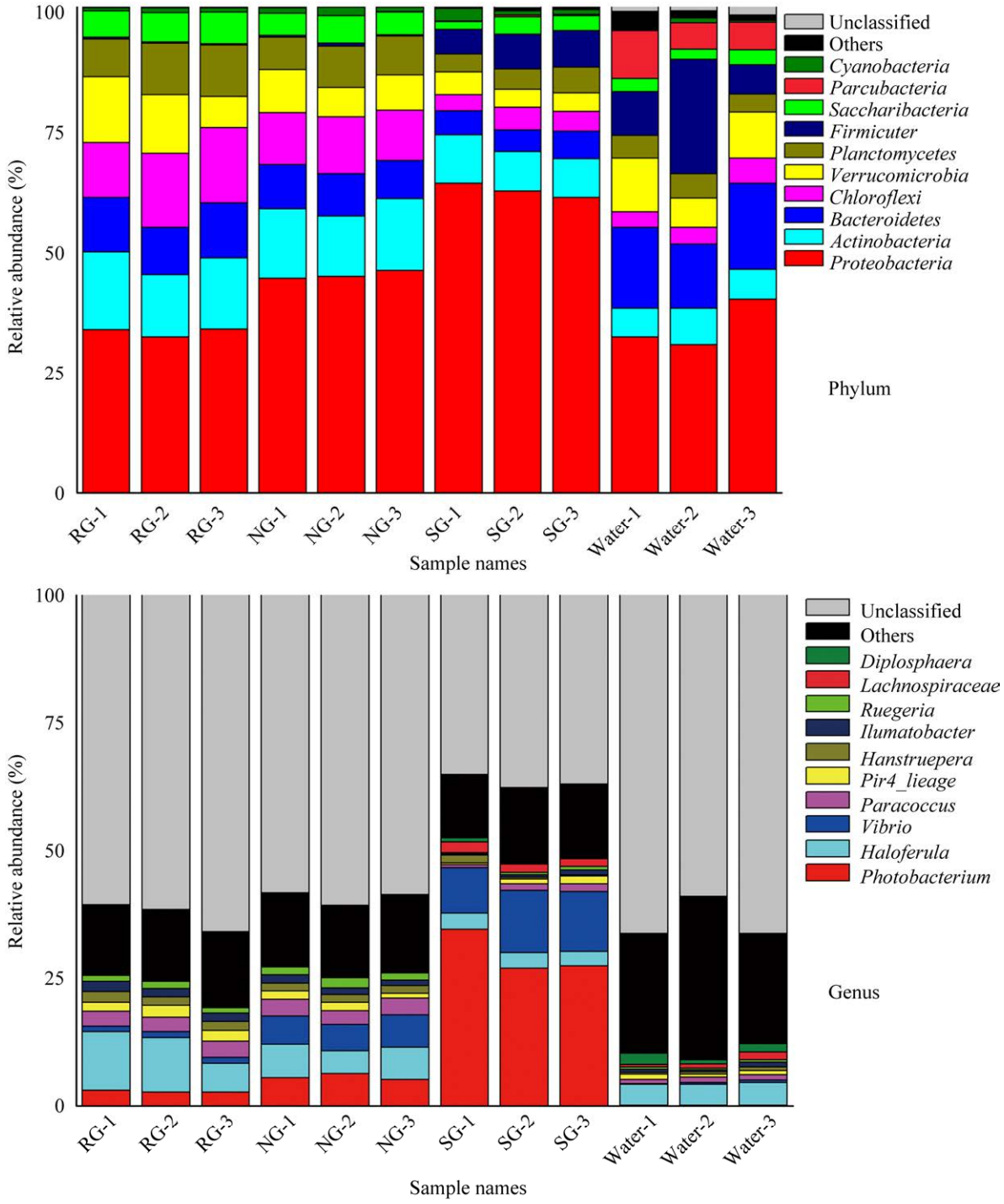


Figure 1 Taxonomy stack distribution of phylum and genus.

2.3 Variation in bacterial communities across shrimp categories

Alpha diversity was used to analyze the complexity of sample species complexity. The bacterial community richness was indicated by the Ace index and the Chao index (NMDCX0000175,

Table S4). And the bacterial diversity was expressed by the Shannon index, the Simpson index, and the Good's coverage. The results showed that there was no significant difference in the Chao index among the three groups (P=0.441) (NMDCX0000175, Figure S2). The alpha diversity was further tested by comparing the Shannon index among the groups using the Kruskal-Wallis test (NMDCX0000175, Table S5) and its differences existed among three groups (P=0.016) (Figure 3).

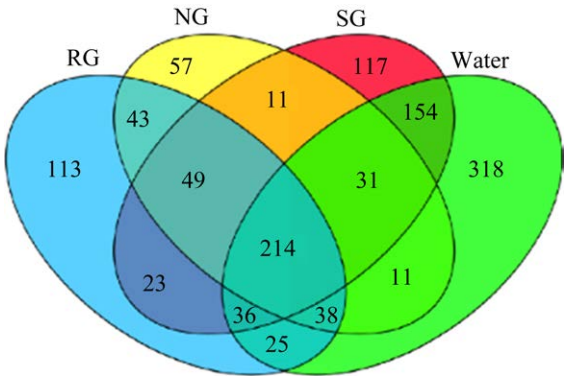


Figure 2 Venn diagram of OTU distribution between different nitrite grows groups. Numbers within compartments indicate OTU counts of according to mathematical sets. Different colors represent sharing between different groups, respectively.

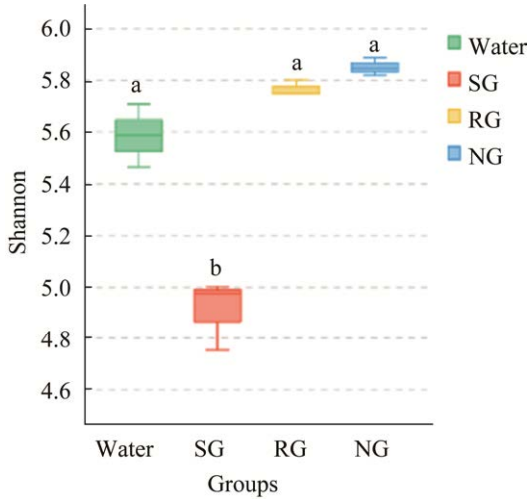


Figure 3 Shannon indices of bacterial communities in each group.

However, comparison between every two groups using t-test, indicated that there was no difference between RG and Water (P=0.116), and between NG and Water (P=0.054), suggesting that the intestinal bacteria of RG and NG group were closer to the water environment. These results indicate that the difference in the growth rate of *L. vannamei* in a high nitrite environment might be caused by the differences in their gut bacterial diversity.

To further investigate the differences in gut microbial community composition, a weighted UniFrac distance matrix was used for PCoA analysis (NMDCX0000175, Table S6). Overall, the bacterial communities differed more between groups with more different growth rates. As shown in Figure 4, the four groups were clearly separated, and the samples in the groups were well aggregated. PCo1 accounted for 58.59% of the difference in PCoA sample difference, and PCo2 accounted for 33.68%. RG and Water were grouped along with PCo1. On the right side of the graph, RG and NG were close in both coordinates, which indicated the RG and NG samples were similar. These results indicated that the bacterial population in each group had a high aggregation, and that the presence of two distinct flora resulted in a change in growth rate. The results showed that the RG and NG had a similar function of intestinal microbial communities.

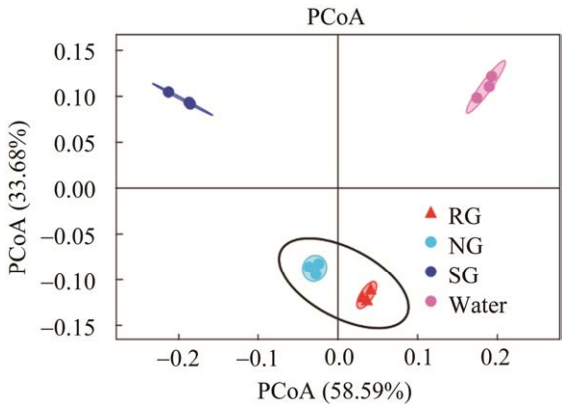
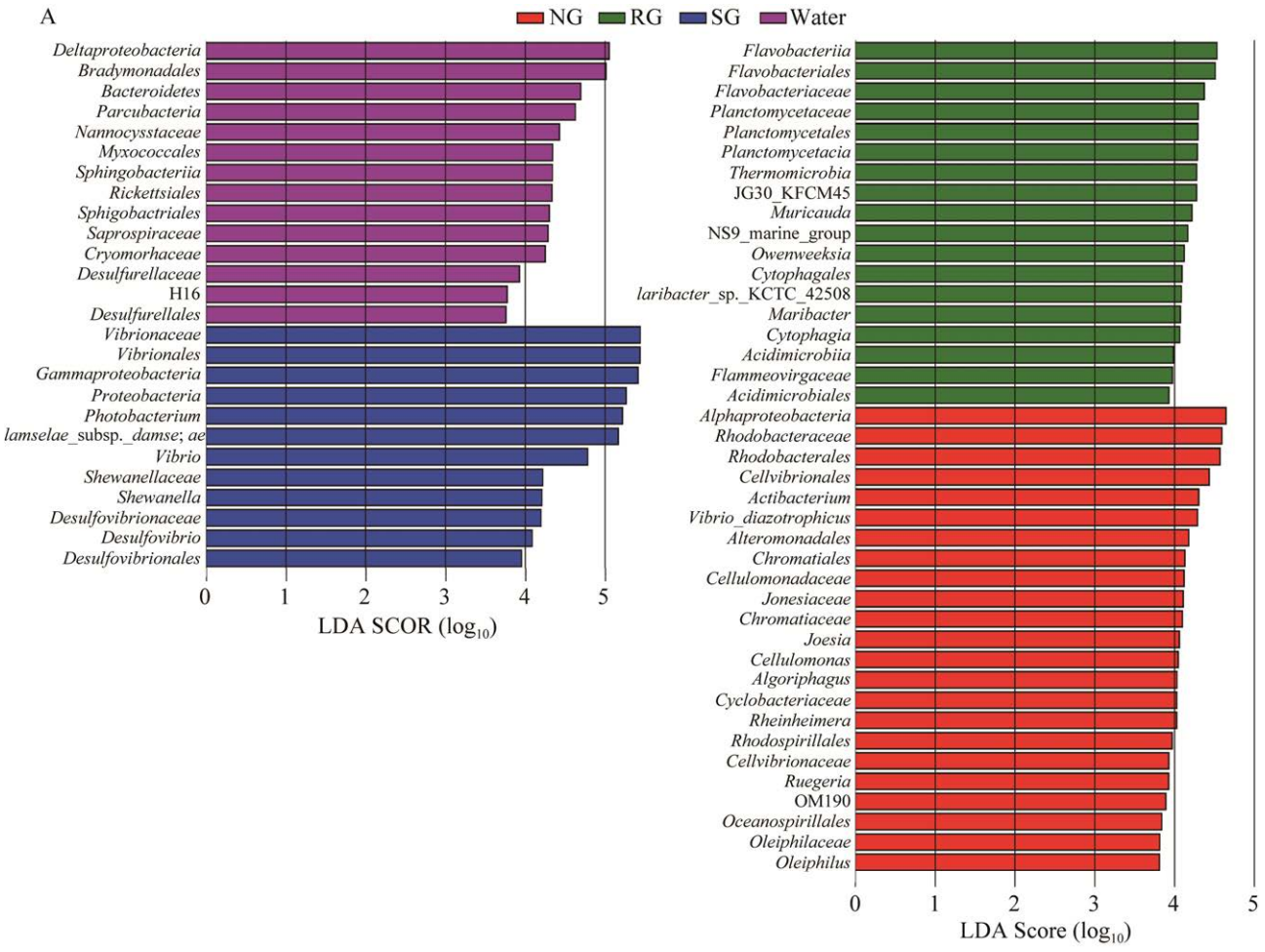


Figure 4 PCoA scores based on the relative abundances of OTUs in all samples.

2.4 Identification of key intestinal bacterial families between groups

Given that intestinal bacterial communities were different among the groups, study was performed to identify which communities characterize these differences. It has been shown that ecological cohesion is highest at the bacterial family level, which can accurately indicate the health of the shrimp[19]. The Kruskal-Wallis test and Wilcoxon test were performed among samples of all groups by using the LEfSe software to perform. The final selected difference was sorted using linear discriminant analysis (LDA) to obtain the left image (Figure 5A). Species with significant differences in the abundance of different groups had been shown, and the length of the histogram represented the magnitude of the impact of different species (LDA score). The evolutionary branch map was then obtained by mapping the differences to the classification tree of the known hierarchy (Figure 5B). The results showed that *Flammeovirgaceae*, *Flavobacteriaceae*, and *Planctomycetaceae* had a greater impact on rapid growth, while *Desulfovibrionaceae*, *Shewanellaceae*, and *Vibrionaceae* had an effect on slow growth. High abundance of *Rhodobacteraceae* was present in the normal growth group. These species had a significant impact on growth. Therefore, these indicator taxa can be used to evaluate shrimp growth.



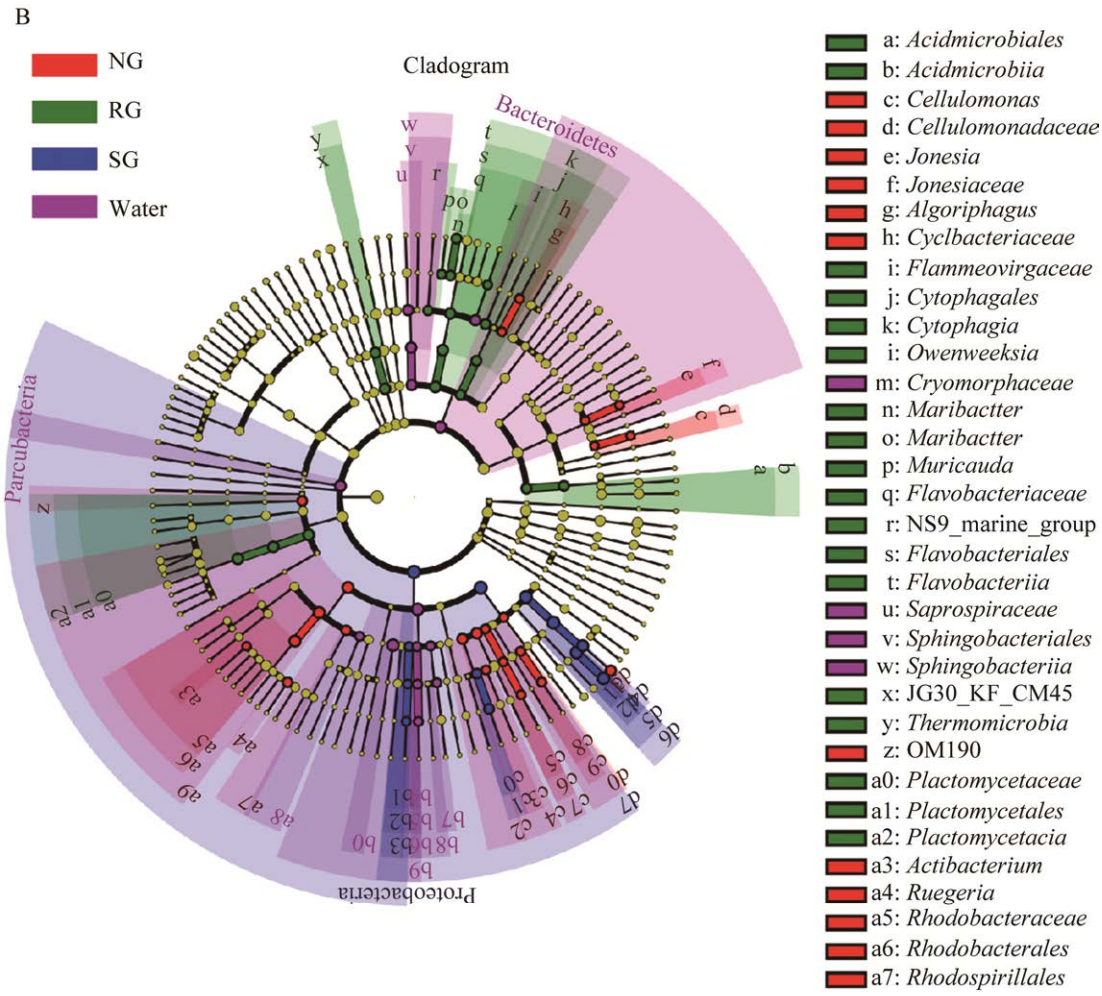


Figure 5 Analysis of taxonomic relative abundances using LEfSe analysis showed that multiple bacterial taxa were differentially enriched among groups. A: Significant differences in the abundance of different groups using LDA. B: The evolutionary branch map by mapping the differences to the classification tree of the known hierarchy.

2.5 Changes in functional composition among the shrimp groups

A variety of evidence indicated that the microbial community functional composition was more closely related to the environment. Tax4fun was used to predict the community function. Compared with RG and NG, nitrogen-related metabolic pathways such as nitrogen metabolism, oxidative phosphorylation, and aminoacyl-tRNA biosynthesis pathways were very low in SG, while two-component system pathways were highly abundant (Figure 6), two-component system allows bacteria to sense, react, and adapt to changes of environment or intracellular state. This may explain that the shrimp in the SG group were more sensitive to changes in ammonia nitrogen and experienced the reduced ability of N metabolism, thereby affecting the growth rate. This may be the main reason for the variance of growth rates of shrimp raised in high nitrous water quality. In addition, the fructose and mannose metabolism pathways were higher in the RG group than those in the other groups (Figure 6).

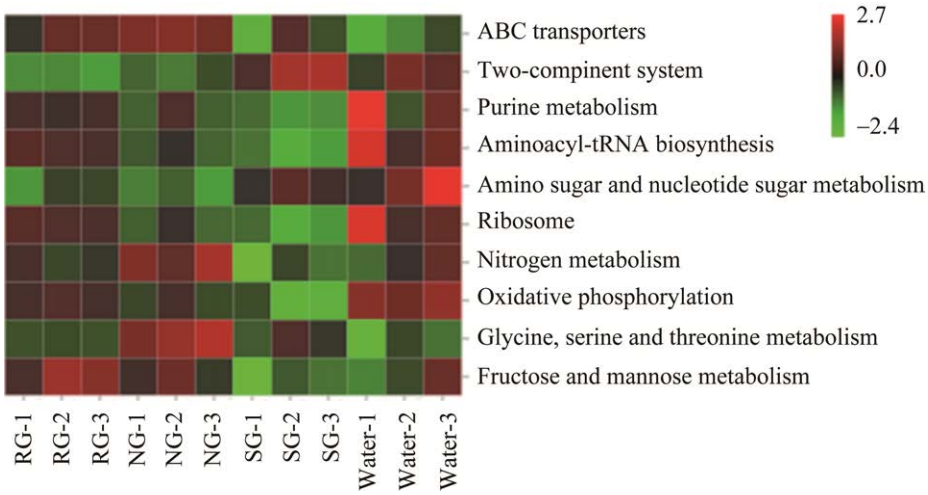


Figure 6 Heatmap of KEGG pathway in different groups of intestinal bacterial.

3 Discussion and Conclusion

In this study, the taxonomic analysis showed that the bacterial community structure of the RG and NG groups of microbes was quite similar, and *Proteobacteria* was the most abundant phylum with an average ratio of more than 30%. The other five most abundant phyla were *Actinobacteria*, Bacteroidetes, *Chloroflexi*, *Verrucomicrobia*, and *Planctomycetes*. *Bacteroidetes*, *Planctomycetes*, and *Actinobacteria*. They have long been recognized as the main phylum in shrimp farming ecosystems[19]. Cluster analysis of 16S rRNA gene sequencing data revealed that some OTUs were shared in the gut of *L. vannamei*. This study was focused on the part of the shared species. The results showed that there was a difference in community diversity among the groups. The diversity of RG and NG groups was significantly higher than that of SG group. Previous studies concluded that the difference in diversity might result in different growth rates of shrimp under a high nitrite environment. Previous studies have also found that, as a typical biological nitrification system, the flora of key processes of nitrogen cycling in shrimp pond water environment was rich in diversity[20]. PCoA analysis showed that the intestinal community composition of NG was closer to RG than SG, which is consistent with our previous studies[21], might be explained by a gradient in the rapid growth and slow growth changes. Then, the major bacterial populations between the groups and analyzed the possible relationship between species composition and growth rate in the shrimp intestines were examined. *Flammeovirgaceae* helps the digestion of sugars and proteins[22]. *Planctomycetaceae* can degrade inorganic salts and plays an important role in the microbial nitrogen cycle[23-24]. Both two bacteria had the greatest impact on RG shrimp. *Acidimicrobiales* are beneficial for biomineralization and the use of multiple energies[25] and *Thermomicrobia* in *Chloroflexi* can degrade carbohydrates and amino acids[26]. The two bacteria above also had an impact on the RG group. This further illustrates that the intestinal microbiota diversity caused growth differences, and the above-mentioned intestinal microbiota can be considered as potential probiotics. *Desulfovibrionaceae*, which can infect the host to cause intestinal proliferation[27] and *Shewanellaceae* can promote fish spoilage[28]. *Vibrionaceae* is closely related to the severity of shrimp disease. Those three bacteria had the highest abundance in SG. These species may have contributed to the slow growth of *L. vannamei*. *Rhodobacter*, a beneficial bacterium, may play a role in inhibiting and killing pathogenic bacteria,

and it was significantly enriched in the NG group. Consistently, substantial inter-individual variations in the gut bacterial composition had been observed in cohabiting larval Atlantic cod[29] and juvenile sea cucumbers[14]. Therefore, changes in the abundance of these indicators may indicate the growth of the shrimp. The growth rate variance drives by bacterial defined here can provide a new opportunity for shrimp farming.

High nitrite causes different growth rates of *L. vannamei*. Given the key functions of the gut microbiota coding and conferring[30], it is easy to speculate that the bacteria in the slow-growing shrimp gut can't alleviate the pressure of high nitrite. Previous studies showed that with normal shrimps, the metabolic pathways divergently changed between overgrown and retarded shrimps, while other pathways were less altered in overgrown shrimps[31]. Consistently, in slow-growing shrimp, the abundance of pathways associated with nitrogen metabolism decreased significantly, while the opposite pattern appeared in the other two groups. It is safe to say that the difference in intestinal flora caused the difference in nitrogen metabolism ability, which affected the ability of shrimp to adapt to the high nitrite environment. Sequentially these differences can explain why the growth rates are different. In addition, studies have shown that interspecies interactions determine the microbial-mediated function[32-34]. The mechanism of interaction between intestinal flora is still unknown. So, further research is needed to be done to clarify the mechanism.

To the best of our knowledge, this is the first attempt to explore a direct link between gut microbiota and shrimp growth rates in a high nitrite environment. This study found that changes in the intestinal bacterial community led to changes in N metabolism, which affected the growth rate of shrimp. The difference in the gut bacteria combination of fast-growing and slow-growing shrimp led to functional changes that caused different adaptations to high nitrite environments, but the mechanism of bacterial flora combination remains to be studied.

In summary, these findings have shown that intestinal flora diversity causes different growth rates in a high nitrite environment, and thus provided a basis for the development of intestinal microbial management strategies for shrimp culture.

Data availability statement

The all data used to support the findings of this study are included within the article.

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