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Microbiota in monocultured *Litopenaeus vannamei* vs. polyculture with *Trachinotus ovatus*

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Abstract

The structures of the microbial community in the intestine, aquaculture water, and sediment of *Litopenaeus vannamei*, both in monoculture and mixed culture with *Trachinotus ovatus*, were analyzed by sequencing 16S rRNA amplicons. 1,120,500 valid reads were obtained from 21 samples, and 3,767 operational taxonomic units (OTUs) were classified. In the two culture modes, the abundance and diversity of bacterial in the sediment were significantly higher than in the *L. vannamei* intestine under the monoculture mode, in the water and intestines of *L. vannamei* and *T. ovatus* under the mix-culture mode ($P < 0.05$). There was no significant difference between the intestinal flora structures of *L. vannamei* and *T. ovatus* in the monoculture mode ($P > 0.05$). The dominant phyla in the sediment under two culture modes were Proteobacteria, Bacteroidetes, and Chloroflexi. The microbial community structure in the water and *L. vannamei* intestine were similar in both culture modes. The dominant phyla included Cyanobacteria, Proteobacteria, and Actinobacteria, with their abundances ranging from 80.88% to 97.10%. Proteobacteria was the dominant phylum in each group of samples, and the dominant genus in both culture modes was *GpIIa*. There was little difference in microbial community structures under the two culture modes; while the culture mode did not affect the core phyla/genera, there were differences in relative abundance. The experimental results provide a reference for the exploration of efficient and specific probiotic screening and microbial formulation techniques.

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Introduction

The whiteleg shrimp, *Litopenaeus vannamei*, is one of the world's leading farmed shrimp species. It has rapid growth, disease resistance, a delicious taste, and high economic benefits. With the improvement of aquaculture technology, aquaculture's scale and stocking density is expanding, and the level of toxic substances in the water will continue to rise. The composition of the flora in the water and environmental factors can affect the diversity of the intestinal microbiota of aquatic animals. When the self-purification capacity of the aquacultural system is exceeded, long-term exposure of aquatic animals to the slow stress of the water environment induces a reduction in the function of the physical intestinal barrier (Duan et al., 2018), growth retardation (Ciji and Akhtar, 2021), triggers stress reactions (Ruvalcaba-Márquez et al., 2021) and damages the immune system (Wang et al., 2020). The deterioration of the farming environment and the frequent outbreak of diseases are becoming more and more prominent (Flegel et al., 2009). To reduce economic losses, farmers often use antibiotics and other drugs to resist disease invasion, which not only destroys the microbiota of the aquatic animal intestine but also easily triggers the resistance of pathogenic bacteria, which seriously restricts the sustainable development of aquaculture and damages human health (Sun et al., 2020).

The main types of *L. vannamei* farming are monoculture or in combination with fish, shellfish, or crab farming. Different farming modes require different aquacultural environments. The aquaculture environment is closely related to the intestinal microbiome of aquatic animals (Chen et al., 2021; Sun et al., 2016). Aquatic animals are in direct contact with water, exchanging materials and information with the outside world. The microflora in the farming environment plays a key role in regulating the homeostasis of aquatic animals' intestinal environments, which promotes growth, nutrient metabolism, and immunity (Li et al., 2018). Although monoculture and mixed culture modes have been studied in recent years, there are few studies on mixed cultures of *L. vannamei* and the pompano fish (*Trachinotus ovatus*) and the diversity of the bacterial communities in their intestines, culture sediment, and water. In this study, 16S rRNA high-throughput sequencing was used to compare the bacterial diversities in the intestine and aquaculture environment of *L. vannamei* cultured under two modes. The aim was to reveal the structural composition of the intestine and aquaculture environment and to explore healthy culture modes for *L. vannamei*, efficient and specific screening of potential probiotic bacteria, and the development of microbial formulations.

Materials and Methods

Sources of samples

The experiment was conducted at Zhanjiang South Coast Fisheries Co., Ltd. is a high-level pond with an area of about 0.67 hm^2 and a water depth of 2.0–2.5 m. Two culture modes were tested: 1) an *L. vannamei* monoculture and 2) *L. vannamei* mixed with *T. ovatus*. Three parallel groups were used for each mode, using culture densities of *L. vannamei* and *T. ovatus* of 7.5×10^5 ind/hm^2 and 6×10^3 tail/hm^2, respectively.

Sample collection and processing

Intestinal, water, and sediment samples from *L. vannamei* and *T. ovatus* were collected on 6 December 2020 and cultured for 92 d. Salinity and pH were measured in situ for each culture group and ranged from 16 to 22 ppt and 8.27 to 8.67, respectively. Culture water was collected by water harvesters in the middle and around the pond's perimeter and mixed into a single sample, with the samples denoted W1, W2, and W3 (monoculture mode) and HW1, HW2, and HW3 (mixed mode). sediment samples were collected at five points 10 cm from the surface and are denoted as S1, S2, and S3 (monoculture mode) and HS1, HS2, and HS3

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(mixed mode). Healthy *T. ovatus* and *L. vannamei* were selected and placed on sterile trays with ice to collect intestinal contents. These were first rinsed with water, swabbed with 75% alcohol, collected with sterile scissors and forceps, and labeled as I1, I2, and I3 (monoculture mode) and HI1, HI2, and HI3 (mixed mode). The average length and weight of 30 *L. vannamei* from each mode were 12.76 ± 1.01 cm and 13.53 ± 3.23 g (monoculture mode) and 11.97 ± 0.71 cm and 10.90 ± 1.86 g (mixed mode), respectively. The average weight of *T. ovatus* was 443.27 ± 73.10 g (n=12). After collection, the water and sediment samples were quickly placed in a bubble chamber with bio-ice packs. The intestine samples were mixed, placed in lyophilization tubes, and temporarily stored in liquid nitrogen tanks. Immediately returned to the laboratory for storage at −80 °C until use.

Extraction of sample DNA and sequencing of 16S rRNA amplicons

Total DNA extraction and high-throughput sequencing of the water, sediment, and intestine samples were conducted at the Beijing Genomics Institute (BGI, Wuhan, PR China). The extracted total DNA was used as a template for amplifying the variable regions V3~V4 of the 16S rRNA gene with amplification primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-TACGGCTACCTTGTTACGACTT-3'). After library construction, the libraries that passed the assay were subjected to high-throughput sequencing on an Illumina HiSeq platform.

Analysis of data

The raw sequences were filtered by offline data and spliced with FLASH (v1.2.11) (Magoč and Salzberg, 2011) software to obtain the tags of highly variable regions. The spliced tags were clustered into different OTUs according to 97% sequence similarity using Usearch (v7.0.1090) (Edgar, 2013) software and compared with the database and species annotations, while the samples were analyzed based on the OTUs and annotation results. High-throughput sequencing data analysis, alpha and beta diversity analyses, and LEfSe analysis of samples from each group were performed based on the OTU and annotation results.

Results

Analysis of high-throughput sequencing data

After sequence optimization, 1,120,500 valid reads were obtained for the 21 samples. All sequencing reads presented in this study can be found in the Short Read Archive (SRA) and accessed under the accession number PRJNA842546 (https://www.ncbi.nlm.nih.gov/sra). The valid sequences were clustered by 97% similarity to divide them into 3767 OTUs. The Venn diagram in **Figure 1B** shows that the total number of OTUs for each group of samples was 119. The number of OTUs obtained from the sediments was much greater than those from the intestine and culture water in both modes, with 104, 214, and 1225 OTUs specific to the *L. vannamei* intestine, water, and sediment, respectively, in the monoculture mode. The number of OTUs specific to the *L. vannamei* intestine, *T. ovatus* intestine, water, and sediment in the mixed culture mode were 64, 197, 63, and 1781, respectively. Analysis of the dilution curves (**Figure 1A**) shows a flat trend for each sample curve, indicating that more data volumes were sequenced and that the variety of OTUs would only increase by a small amount, indicating that the results from these sequencing data are reasonable (**Table 1**).

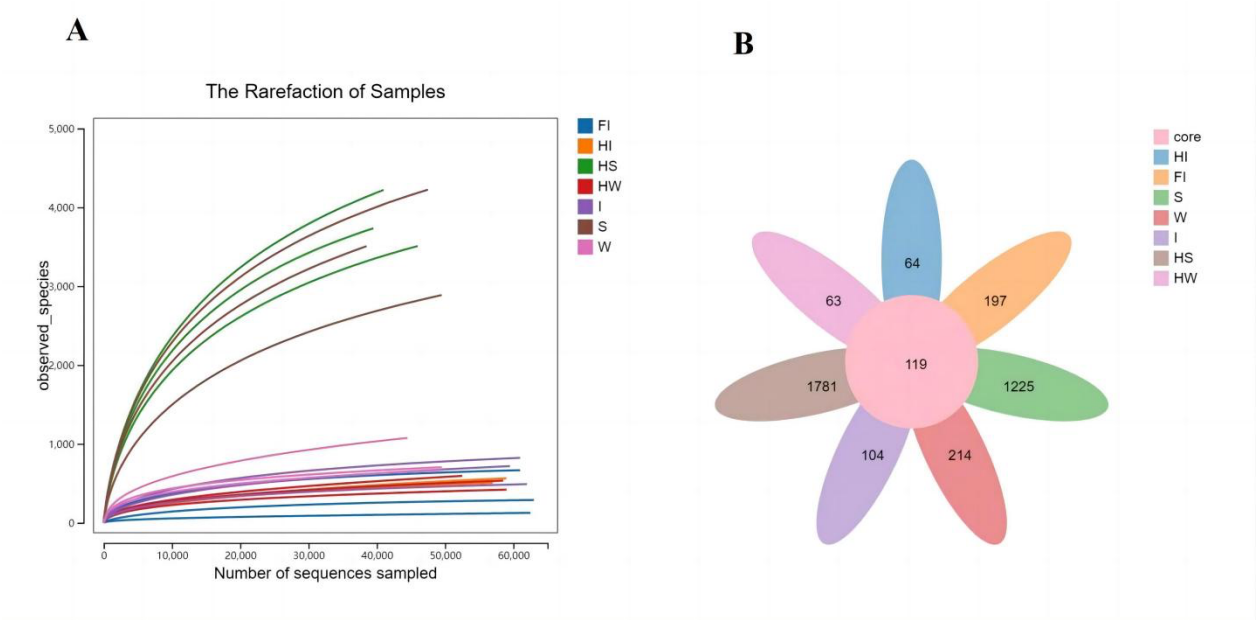

Figure 1 OTU analysis of bacterial diversity: (A) Multi-sample rarefaction curves; (B) Venn diagram.

Table 1 High-throughput sequencing analysis of each group of samples under the two culture modes. Each value (mean ± SD) represents a mean of three replicates. Within each row, entries with the same letters indicate a non-significant difference ($P > 0.05$).

Group	*Tags*	*OTUs*	*Coverage*
FI	62483.67 ± 1113.86^{a}	359.67 ± 277.53^{b}	0.9986 ± 0.0006^{a}
HI	57339.33 ± 1830.52^{a}	539.00 ± 29.82^{b}	0.9962 ± 0.0005^{a}
HS	42281.00 ± 3380.18^{b}	3824.00 ± 369.01^{a}	0.9718 ± 0.0047b^{a}
HW	56838.67 ± 3611.85^{a}	516.00 ± 89.37^{b}	0.9965 ± 0.0014^{a}
I	60996.33 ± 1262.44^{a}	677.00 ± 171.22^{b}	0.9967 ± 0.001^{a}
S	45386.33 ± 5612.09^{b}	3545.00 ± 670.8^{a}	0.9751 ± 0.0054^{b}
W	48174.67 ± 2784.43^{b}	817.67 ± 227.04^{b}	0.9943 ± 0.0028^{a}

Alpha diversity analysis of the flora of different culture modes

Alpha diversity analysis showed that the Chao and Ace indices of the two-mode sediment groups were higher than those of the *L. vannamei* and *T. ovatus* intestine and water, indicating that the abundance of bacterial flora was significantly higher in the sediments than in other samples ($P < 0.05$), but there was no significant difference between the different mode sediment groups ($P > 0.05$). Simpson's and Shannon's indices were used to analyze the microbial species diversity, with smaller Simpson's and larger Shannon's values indicating higher diversity. The Shannon index of the mixed sediment group was the largest (6.59 ± 0.22). In comparison, the *T. ovatus* intestine group was the smallest (1.6 ± 1.68), indicating that the mixed sediment group had the highest bacterial diversity and the *T. ovatus* intestine group had the lowest (**Table 2**).

Table 2 Bacterial diversity indices between groups in the two culture modes. Each value (mean ± SD) represents a mean of three replicates. Within each row, entries with the same letters indicate a non-significant difference ($P > 0.05$).

Group	*Chao*	*Ace*	*Shannon*	*Simpson*
FI	455.85 ± 236.28^{b}	513.83 ± 221.64^{b}	1.6 ± 1.68^{c}	0.55 ± 0.423^{a}
HI	825.41 ± 78.11^{b}	958.39 ± 157.93^{b}	2.9 ± 0.59^{bc}	0.14 ± 0.078^{ab}
HS	4876.07 ± 503.08^{a}	4943.41 ± 516.56^{a}	6.59 ± 0.22^{a}	0.01 ± 0.004^{b}
HW	848.29 ± 279.29^{b}	984.96 ± 401.48^{b}	2.28 ± 1.14^{c}	0.39 ± 0.297^{ab}
I	882.84 ± 228.10^{b}	873.97 ± 221.07^{b}	3.08 ± 0.34^{bc}	0.17 ± 0.039^{ab}
S	4566.71 ± 747.70^{a}	4641.9 ± 754.77^{a}	6.43 ± 0.61^{a}	0.01 ± 0.01^{b}
W	1168.62 ± 372.28^{b}	1272.43 ± 612.38^{b}	4.02 ± 0.18^{b}	0.06 ± 0.012^{b}

Beta diversity analysis of flora under different culture modes

A principal coordinates analysis was carried out using Weighted Unifrac distances further to visualize the differences in species diversity between samples. The horizontal axis PCoA1 contributed 45.41% to the sample, while the vertical axis PCoA2 contributed 20.56%. The proximity of the monoculture sediment group to the mixed sediment group suggests that the flora species were most similar between the two sediment groups. Still, the similarity in flora structure to all other groups of samples was low. The distance between the *L. vannamei* intestine and the water in the monoculture mode was relatively close to the distance between the water and the *L. vannamei* intestine in the mixed mode, indicating that the microbial community structure of the *L. vannamei* intestine and water in the monoculture mode is more similar to that of the water, *L. vannamei* and *T. ovatus* intestine in the mixed mode (**Figure 2**).

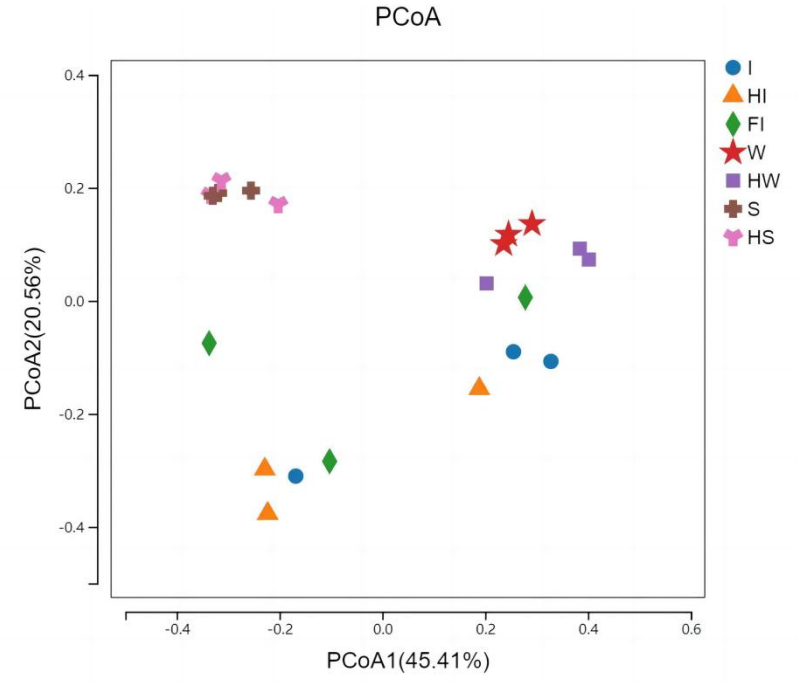

Figure 2 PCoA analysis graphs for all samples in both modes.

UPGMA hierarchical clustering analysis was done on each sample using the Weighted Unifrac distance matrix. The results of the clustering of each sample at the gate level were integrated into a species abundance histogram, which was used to determine the species composition and variability between samples. The samples from the sediment group in the two modes were clustered into one group with a similar community structure, and the two sediment groups had higher abundances of Proteobacteria, Bacteroidetes and Chloroflexi, while the *L. vannamei* intestine group and the water flora in the monoculture mode had clustering results of the same cluster with different branches and the same cluster with the same branches as the water flora and the *L. vannamei* and *T. ovatus* intestine group in the mixed culture mode, indicating that the structural composition of the five groups was similar and differed from the sediment bacterial community structure, which is similar to the alpha diversity analysis (**Figure 3**).

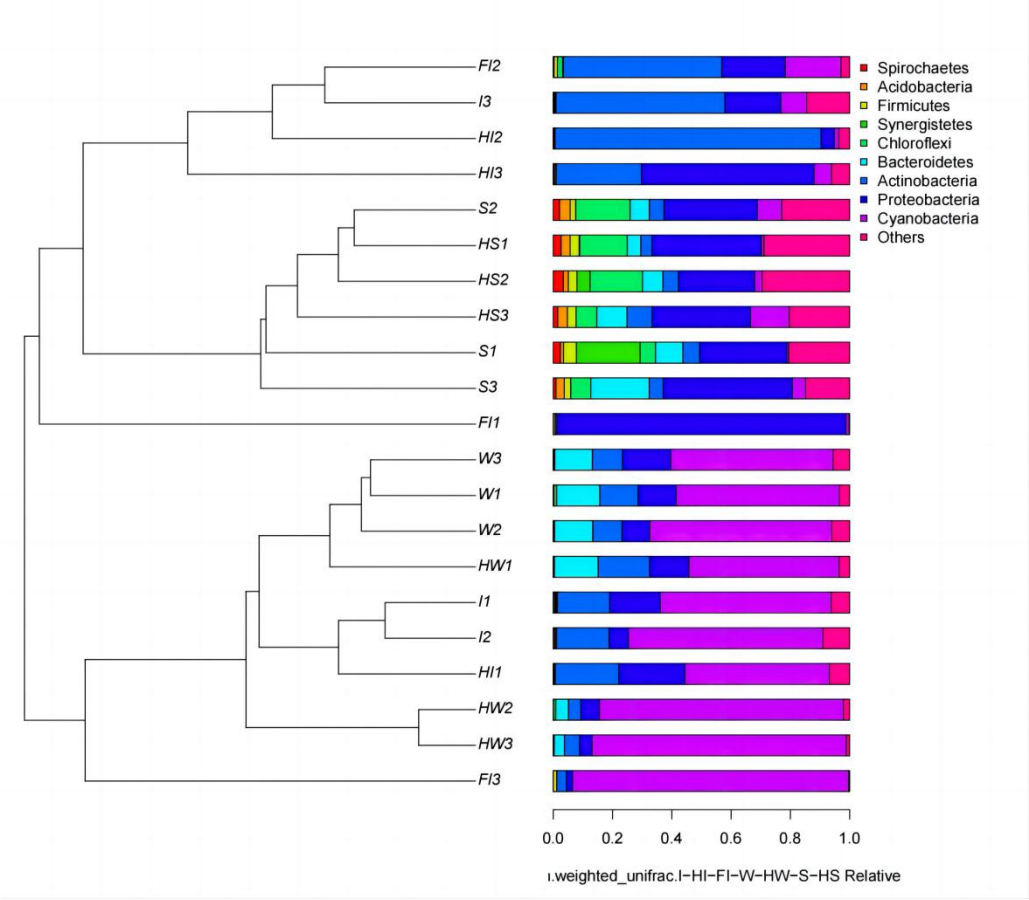

Figure 3 UPGMA clustering tree with clade-level histograms of the colony structure of all samples in both culture modes.

The species compositions of the main bacterial groups in the intestine, sediment, and water of *L. vannamei* and *T. ovatus* at the phylum and genus levels were analyzed for each culture mode. Species with abundances < 0.5% in all samples and those not annotated at this taxonomic level were combined into the group "Others." Cyanobacteria, Actinobacteria, and Proteobacteria were the dominant phyla in the monoculture intestine and the mixed *L. vannamei* and *T. ovatus* intestine, with a combined abundance of 88.91–97.10%. The proportion of diversity levels in the sediment group was significantly higher than in all other groups of samples in both modes. The differences in the structure of the bacterial groups in

the two sediment groups were small, with the dominant phyla in the monoculture sediment group being Proteobacteria (34.23%), Bacteroidetes (11.30%), Chloroflexi (10.22%) and Synergistetes (7.86%). The dominant phyla in the mixed sediment group were Proteobacteria (31.67%), Chloroflexi (13.67%), and Bacteroidetes (7.25%). The dominant phyla in the monoculture water group were Cyanobacteria (57.08%), Chloroflexi (13.36%), Proteobacteria (12.87%), and Actinobacteria (10.92%). The dominant phyla in the mixed water group were Cyanobacteria (73.72%), Actinobacteria (8.59%), and Proteobacteria (7.72%). From the analysis of the above results, it is clear that there is an overlap in the dominant phyla of the 2 modes, but there are differences in the relative abundance in each sample (**Figure 4**).

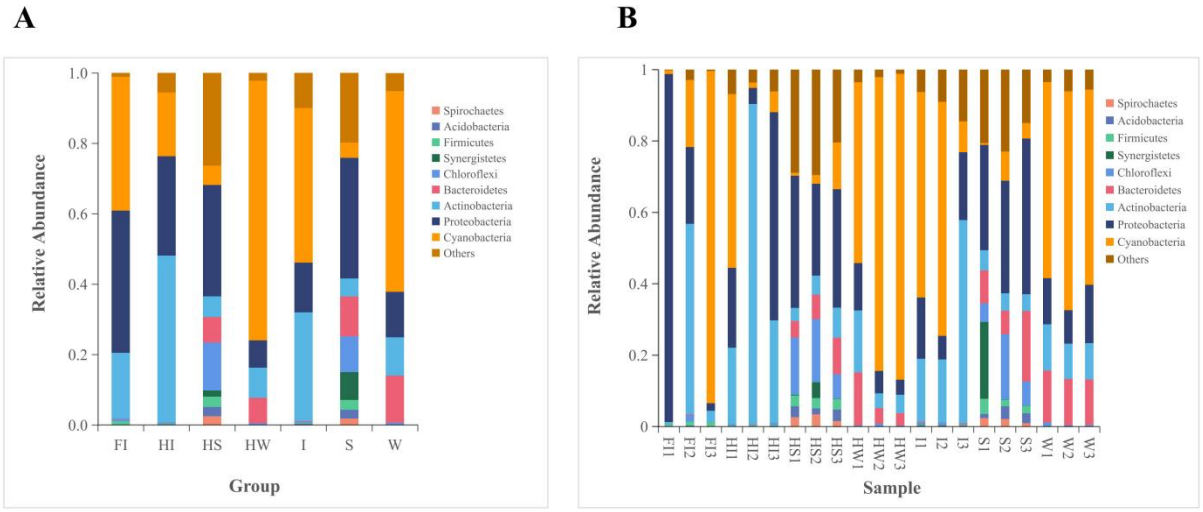

Figure 4 The dominant bacterial community at the phylum level in both culture models, (A) indicates the bacterial community composition of each group, (B) appearing in each sample.

As shown in **Figure 5**, the dominant genera in the monoculture *L. vannamei* intestine group were *GpIIa* (42.43%), *Aquihabitans* (12.12%), and *Ilumatobacter* (2.08%). The dominant genera in the *L. vannamei* intestine of the mixed culture group were *GpIIa* (17.42%), *Aquihabitans* (15.35%), *Ruegeria* (12.89%), and *Ilumatobacter* (2.02%). In comparison, those in the *T. ovatus* intestine were *GpI* (30.47%), *Aquihabitans* (7.05%), and *GpIIa* (4.70%). There were significant variations in the proportions of genera in each sample. The dominant genus in the monoculture and mixed sediment groups was *GpIIa*, with 2.56% and 3.98%, respectively. The dominant genera in the monoculture group were *GpIIa* (41.08%), *Nitriliruptor* (2.73%), and *GpIV* (2.39%), the latter two having significantly higher abundances than in the other groups. The dominant genera in the mixed water group were *GpIIa* (62.98%), *Marivita* (3.33%), and *Phaeodactylibacter* (2.30%). This shows species composition and abundance differences between the two culture modes, with the common dominant genus being *GpIIa*.

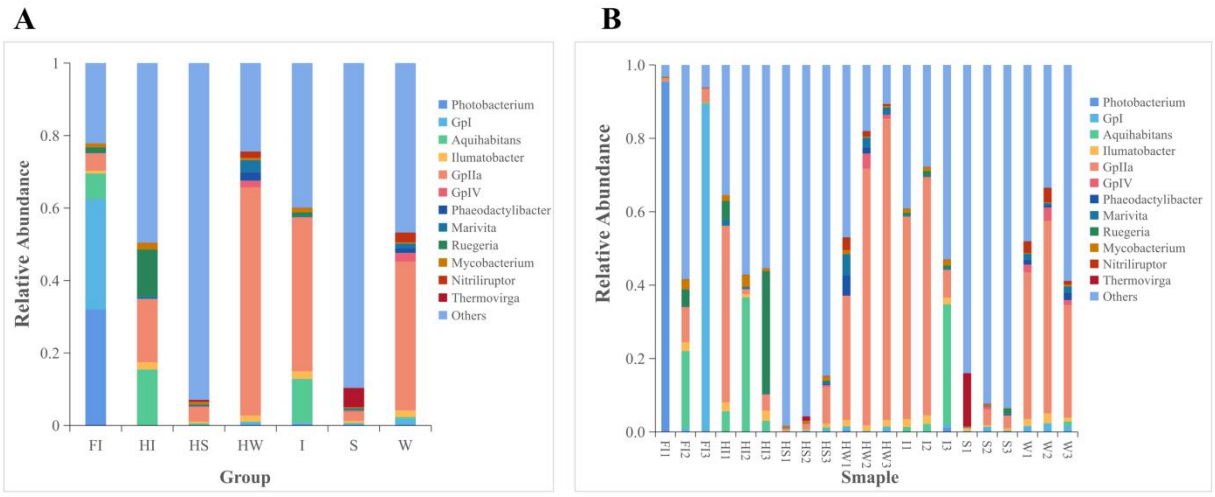

Figure 5 The dominant bacterial community at the genus level in both culture models, (A) indicates the bacterial community composition of each group, (B) appearing in each sample.

Analysis of differences in bacterial flora structures under different culture modes

An LDA analysis of significant differences between the bacterial species in each sample group (LDA score ≥ 2) was conducted. The absolute LDA values were greater than 2 in both the monoculture and mixed water groups and were not statistically different in Biomaker. The clustering tree from LEfSe analysis showed a predominance of differential species (markers colored red and purple). The mixed sediment group had the highest number of significantly abundant differential flora with 39 differential groups, followed by the monoculture sediment group (18), the monoculture water group (4), the mixed *T. ovatus* intestine group (3), and the mixed *L. vannamei* intestine group (2). The above results illustrate that the differences in aquaculture water and intestine flora structure under the 2 culture modes are insignificant and that the sediment group in the mixed culture mode has more different species than the monoculture sediment group (**Figure 6**).

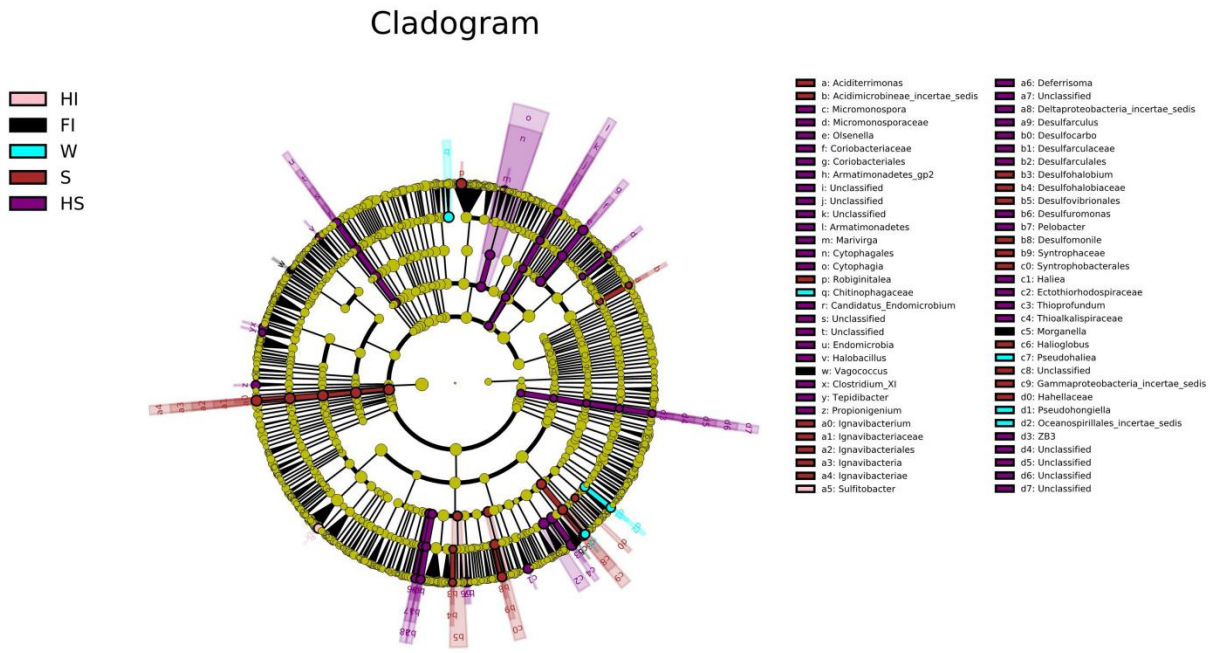

Figure 6 LEfSe analysis of bacterial species with significant differences between samples in the two models.

Discussion

This research analyzed 21 samples from the two-culture mode for bacterial composition and diversity by high-throughput sequencing. The results show that the Shannon indexes of the monoculture and mixed sediment groups were higher than those of samples from the water and intestine. This indicates that the bacterial diversity in the intestines of *L. vannamei* and *T. ovatus* and in the water was lower than that in the sediment. This result is consistent with previous analyses of the intestine bacterial diversity of *L. vannamei* and its aquacultural environment (Sun et al., 2016). Similarly, Sun et al. (Sun et al., 2019) reached consistent conclusions after studying the diversity of microflora in the intestine, water, and sediment of several aquatic animals. Some studies have shown that the intestinal flora structure of aquatic animals is not significantly correlated with that of the aquacultural environment. Alexopoulos et al. (Alexopoulos et al., 2011) studied farmed fish for 20 months and found no remarkable

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correlation between the water and fish intestine microbial communities. Xiong et al. (Xiong et al., 2015) concluded that there were no significant differences in the microbial communities in the intestine and water of *L. vannamei*, regardless of their health status. However, it has also been pointed out that the microbiota in the aquacultural environment is closely related to that of the intestine in aquatic animals (Huang et al., 2018). Song et al. (Song et al., 2020) revealed that the structure of bacterial flora in the intestine and water of Penaeus japonicus was similar to that of the sediment. In this research, the results of PCoA, UPGMA, LDA, and LEfSe analysis showed that the bacterial structure in the water was similar to that in the intestine of *L. vannamei* and *T. ovatus* under both culture modes. This suggests that the bacterial composition of water is likely to influence the micro-ecological compartments of shrimp and fish intestines. This is probably because the *L. vannamei* and *T. ovatus* in this study had a limited living space and were in the aquaculture water environment for a long time. The flora in the water body enters the shrimp and fish via respiration and feeding while their excrement diffuses into the water, creating an interaction between the water body and intestinal flora. In addition, regular water changes were carried out during the culture process to avoid the deterioration of water quality, which can cause a series of diseases. Hence, this made the structure of the intestinal flora similar to that of the water but different from that of the sediment. It has also been suggested that the feed composition (He et al., 2017), environmental factors (Zhang et al., 2021), individual genetic development (Garibay-Valdez et al., 2020), and disease and season (Li et al., 2019) all influence the diversity of intestinal flora in *L. vannamei*. The specific factors influencing this warrant further exploration.

Many investigations have shown that Proteobacteria are commonly found in numerous aquatic animal farming environments (Moschos et al., 2022;Chen et al., 2021). Furthermore, Actinobacteria, Bacteroidetes, and Chloroflexi are the dominant phyla in mariculture, where they play influential roles in the biodegradation of plant and animal remains and organic matter. The Actinobacteria also show antagonistic activity against pathogenic bacteria in shrimp. Cyanobacteria are one of the dominant phyla commonly found in marine areas, which may be due to geographical and environmental conditions. Cyanobacteria are the dominant phylum in the intestinal flora of aquaculture animals, related to the early feeding of aquatic animals on phytoplankton. In the present study, the relative abundances of Cyanobacteria, Proteobacteria and Actinobacteria were significantly large in the intestines of *L. vannamei* and *T. ovatus.* At the same time, Proteobacteria, Chloroflexi, and Bacteroidetes were the dominant phyla in the sediments, and Cyanobacteria, Actinobacteria, Proteobacteria, and Chloroflexi were dominant in the water. From the above results, it is obvious that the Proteobacteria are the core phylum in the intestine, water, and sediment, indicating that they are capable of adapting well to the environmental changes of the different culture modes. Fan & Li et al. (Fan & Li et al., 2019) compared the structure of the intestine and sediment flora of *L. vannamei* with growth performance, finding that the dominant phyla were Proteobacteria, Actinobacteria, Cyanobacteria Bacteroidetes, etc. in all groups. They also studied the microbial communities in sediments and intestines of *L. vannamei* cultured in freshwater and marine environments, finding Proteobacteria to be the dominant phylum in all samples, with relatively high abundances (Fan et al., 2018). Md Zoqratt et al. (Md Zoqratt et al., 2018) studied the microbial compositions in *L. vannamei* and farmed waters from two countries. They found differences in intestinal and water microflora, with Proteobacteria being the dominant phylum in all samples. The above results are similar to those of the present study, which suggests that the core phyla in the intestines of *L. vannamei* and *T. ovatus* and in their environment are not dependent on the culture mode but affect the relative abundance of the dominant core phylum in the samples. The results of the present study are consistent with those of Dong et al. (Dong et al., 2019) regarding the intestinal flora of *Macrobrachium rosenbergii*.

Equally, at the genus level, the dominant genera in the *T. ovatus* intestine were *GpI*, *Aquihabitans,* and *GpIIa*, with the latter being predominant in the intestines of both shrimp

groups. The dominant genus in both the monoculture and mixed sediment groups was *GpIIa*; those in the monoculture group were *GpIIa* and *Nitriliruptor*, and those in the mixed group were *GpIIa* and *Marivita*. The dominant genus in all groups of samples was *GpIIa*, which accounted for 4.70%, 17.42%, 42.43%, 62.98%, 41.08%, 3.98%, and 2.56% in the *T. ovatus* intestine group, the mixed *L. vannamei* intestine group, the monoculture *L. vannamei* intestine group, the mixed water group, the monoculture water group, the mixed sediment group, and the monoculture sediment group, respectively. This suggests that the *GpIIa* genus is more dominant in the intestine and water of *L. vannamei* and *T. ovatus* than in the culture sediment, indicating a close relationship between the intestinal and water flora.

Potential probiotics are mainly isolated and screened from the intestines of healthy aquatic animals and their breeding environments. The dominant taxa are more competitive and relatively easy to colonize as a probiotic. Research on the dominant taxa can help screen efficient and specific probiotics and develop microbial preparations. Luis-Villaseñor et al. (Luis-Villaseñor et al., 2011) isolated four potentially probiotic Bacillus strains from the intestine of *L. vannamei* that were antagonistic to *Vibrio*; they were able to colonize the intestine and improve survival. Huang et al. (Huang et al., 2018) showed that *Nitriliruptor* was the dominant bacterial genus in the intestine and culture environment of *L. vannamei* and was positively correlated with physiological health indicators. Hence, isolating probiotic *Nitriliruptor* bacteria for shrimp culture is potentially useful. Screening potential probiotics from the dominant bacteria in the aquatic animal intestine and aquacultural environment can help to regulate water quality precisely, improve the aquatic animal's intestinal flora, regulate the micro-ecological internal balance, improve growth rate and autoimmunity (Ninawe and Selvin, 2009; Zuo et al., 2019), which is conducive to promoting sustainable development of aquatic animals.

In this study, the abundance and diversity of sediment flora were vastly greater than those of the intestine and water in the two culture modes. There was no significant difference between the structures of the two groups of sediment flora, with the highest number of OTUs being in the mixed culture mode sediment. The intestinal microbiome of *L. vannamei* is closely related to its culture environment and is more similar in structure to that of the water than that of the sediment. The culture mode had little effect on the intestinal and aquacultural environment flora of *L. vannamei*. The core phylum and genus in all groups of samples were Proteobacteria and *GpIIa*, respectively, although there were differences in their relative abundances.

Acknowledgments

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