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Research Article 研究报告

Bacillus licheniformis affects the bacterial community in the zero-water exchange aquaculture system of *Penaeus vannamei*

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Abstract: [Objective] Despite the extensive studies about the effects of *Bacillus licheniformis* on the immune response, disease resistance, and nutrition of *Penaeus vannamei*, little is known about the effects on the intestinal and environmental microbial communities of *P. vannamei* in the zero-water exchange aquaculture system. **[Methods]** The intestinal, seawater, and sediment samples were collected from the environment supplemented with *B. licheniformis* in food or water for 16S rRNA gene sequencing and linear discriminant analysis effect size (LEfSe). **[Results]** Adding *B. licheniformis* had little effect on the growth of *P. vannamei*, and different adding methods had little effect on the intestinal microflora. However, the addition of *B. licheniformis* changed the intestinal microbial community and improved the immunity of *P. vannamei*. **[Conclusion]** The findings help us to comprehensively understand the changes in shrimp intestine and environment after *B. licheniformis* is added in feed and water in the zero-water exchange aquaculture system, thereby providing basic information for choosing the right probiotics and addition ways to sustain the health of *P. vannamei*.

Keywords: *Penaeus vannamei*; bacterial function; bacterial community; *Bacillus licheniformis*; zero-water exchange aquaculture system

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地衣芽孢杆菌对南美白对虾零水交换养殖系统细菌群落的影响

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摘　要：【目的】地衣芽孢杆菌(*Bacillus licheniformis*)对南美白对虾(*Penaeus vannamei*)免疫反应、抗病性和营养的影响已被广泛研究，但零水交换养殖系统下地衣芽孢杆菌对对虾肠道和养殖水环境微生物群落的影响尚不清楚。【方法】通过收集添加地衣芽孢杆菌在饲料或水中后，对虾肠道、池水和池低沉积物样品，通过16S rRNA基因测序和线性判别分析(linear discriminant analysis effect size, LEfSe)进行微生物分析。【结果】结果表明，添加地衣芽孢杆菌对对虾的生长影响较小。此外，添加方式的不同对对虾肠道菌群的影响较小。但添加地衣芽孢杆菌可以有效地改变对虾肠道微生物群落，并改善对虾免疫力。【结论】这些结果有助于全面了解在零水交换养殖系统中，通过饲料和水添加地衣芽孢杆菌后对虾肠道和环境的变化，从而为选择正确的益生菌以及如何添加益生菌维持对虾健康提供基础信息。

关键词：南美白对虾；细菌功能；细菌群落；地衣芽孢杆菌；零水交换养殖系统

With the improvement of living standards and increasing requirements for nutritional diversification[1], people's demand for fish and shrimp has increased year by year[2-3]. *Penaeus vannamei* is native to the Pacific coast of south America[4-5]. By virtue of its high nutritional value[6], a wide range of salinity adaptation[4], high temperature tolerance[7] and strong stress resistance, *P. vannamei* has become a worldwide cultured species[8-9]. However, since 2010, the production of *P. vannamei* has been declining[10-11]. On the one hand, due to the water quality damage caused by environmental pollution, such as ammonium and nitrite in water exceeding standards[12]. On the other hand, various diseases caused by pathogens are gradually increasing, such as acute hepatopancreatic necrosis disease (AHPND) and white spot syndrome virus[13-14]. Therefore, improving the quality of water for shrimp culture and the ability of shrimp to resist various pathogens are urgent problems to be solved in shrimp culture.

In the aquaculture industry, antibiotics were once the first choice for people to deal with various diseases[15]. However, with the increase in antibiotic use, people have gradually realized the

disadvantages caused by the use of antibiotics: pathogens can produce drug resistance[16], disrupt ecological balance, and produce drug residues in aquatic products. Therefore, people began to choose a way that can replace antibiotics. Probiotics are considered to be one of the good substitutes for antibiotics[17-18]. It can improve the health of the host by adjusting the balance of the host's intestinal flora, therefore has gradually become the alternative to antibiotics[19-20]. *Bacillus licheniformis* is one of the probiotics widely used by people. Previous studies showed that *Bacillus licheniformis* can regulate water quality[21], promote growth[22], and increase digestive enzyme activity[23]. In this study, a strain of *Bacillus licheniformis* selected in the previous study was added to feed and water respectively to study its effects on the growth and intestinal flora of shrimps.

In the current experiments on the culture of *P. vannamei*, most of the aquaculture system adopted are recirculating aquaculture or water-exchange aquaculture system, and this is also the case in many studies including probiotics[24]. In the process of production, the above aquaculture system will cause problems such as the increase of cultivation cost[25], and the tense utilization of water resources[26]. The zero-water exchange aquaculture system means that there is no water quality exchange with the outside world during the entire aquaculture process, and only the water lost due to evaporation is supplemented[26]. Compared with the traditional aquaculture system, this aquaculture system is not restricted by the region and the natural environment, and the water resource utilization efficiency is high. It is the future standardized aquaculture system[27]. In this mode, the influence of probiotics on the composition and changes of microorganisms in the intestinal and environment of *P. vannamei* can be studied more accurately.

Therefore, we adopted the zero-water exchange aquaculture system and added *Bacillus licheniformis* to feed and water respectively to explore its influence on the microbial community structure in the intestine and surrounding environment of *P. vannamei*, expecting to find the interaction between *Bacillus licheniformis* and related bacteria during the aquaculture process.

1 Materials and methods

1.1 Preparation of experimental diets

The bacteria used in this experiment was *Bacillus licheniformis*, which was selected in the intestine of shrimp that accounted for the first 15% of the body weight in the shrimp farming ponds in the previous study. The strain preserved in the laboratory was first activated. The bacterial strains were cultured in the 2216E medium for 24 h at 37 °C. The cells were harvested after the cultures were centrifuged (5 000×*g*, 4 °C, 10 min) and washed with sterile sea water. The cells were resuspended in sterile sea water before use, and sprayed on basal feed at 1×10^8 colony-forming units (CFU)/g. Finally, feed was dried at room temperature for 6 h and stored at 4 °C.

1.2 Determination of ammonia nitrogen

Concentration of ammonia nitrogen was determined by the indophenol blue method. In briefly, ammonia-N concentration was measured and adjusted every day using HACH ammonia reagent (salicylic acid method, reference manual operation) and DR/850 portable photometer instrument.

1.3 Experiment set-up

P. vannamei, weighing (0.54±0.10) g, were obtained from an aquaculture farm in a suburb of Guangzhou, China. Before the experiment, shrimps were breed in standard shrimp culture ponds (2.8 m×3.8 m). All shrimps were maintained in seawater (salinity, 5‰, pH 8.0±0.2) at (25±2) °C with continuous aeration. And fed three times per day with commercial shrimp feed, the basal diet containing crude protein 40%, crude fat 8%, ash 18%, and water 12%.

Each treatment groups are as follows: the AF group indicated that the feed supplemented with *Bacillus licheniformis* was fed during the experiment; the AW group indicated that *Bacillus licheniformis* was added to the aquaculture water

every seven days during the aquaculture process, the dosage of *Bacillus licheniformis* is 2×10^2 CFU/mL; the C group represents was not added the *Bacillus licheniformis* in feed or water. Triplicate samples were set up for each treatment. Shrimps were randomly assigned to nine buckets (50 cm×38 cm×30 cm) of 30 shrimps each. Each bucket contains 50 L of water. During the experiment, no water exchange is performed, only the amount of water consumed by evaporation is replenished. The AW group and group C were fed commercial feed daily. The commercial feed containing crude protein 40%, crude fat 8%, ash 18%, and water 12%. The daily feeding rate was 10% of the body weight. Shrimp were fed 3 times a day at 8, 14 h and 20 h, respectively.

1.4 Sample collection

After the five-week aquaculture experiment was over, the shrimp and the environment were sampled. Six shrimps were taken from each tank. The surface of the shrimp was wiped with 75% alcohol and the intestine was aseptically dissected. The intestine was put into a 1.5 mL sterile centrifuge tube, frozen in liquid nitrogen and immediately stored at −80 °C. A disposable Pasteur straw was used to collect water samples (15 mL) from the surface, middle and bottom of the water in the aquaculture bucket. The water samples were filtered through the filtration membrane and placed in a 15 mL sterile tube, which was immediately placed in liquid nitrogen for preservation. In the same way, the sediment is sucked out from the bottom of the aquaculture bucket, transferred into a 1.5 mL centrifuge tube, marked and immediately placed in a liquid nitrogen tank for preservation.

1.5 DNA Extraction, amplification, and sequencing

Total microbial DNA was extracted from 27 samples using the HiPure Soil DNA Kit (Magen) according to manufacturer's protocols. The primer pair 341F (CCTACGGGNGGCWGCAG) and 806R (GGACTACHVGGGTWTCTAAT) were used to amplify the 16S rDNA V3–V4 region of the ribosomal RNA gene, and the barcode is an eight-nucleotide sequence unique to each sample. PCR reactions were performed in triplicate as follows: 5 μL of 10× KOD Buffer, 5 μL dNTPs of 2.5 mmol/L dNTPs, 1.5 μL of each primer (5 μmol/L), 1 μL KOD Polymerase, 100 ng template DNA and ddH_2O to a total volume of 50 μL. The PCR reactions were performed on the ABI Gene Amp® PCR System 9700 (Applied Biosystems, Foster City) as follows: 95 °C for 2 min, followed by 27 cycles of 98 °C for 10 s, 62 °C for 30 s, 68 °C for 30 s, and a final extension of 68 °C for 10 min. All PCR products were detected using 2% agarose gels, purified with the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences), quantified using ABI StepOnePlus Real-Time PCR System (Life Technologies). Purified PCR amplicons were pooled in equimolar and paired-end sequenced (2×250) on an Illumina MiSeq sequencing platform at Genedenovo Bioinformatics Technology Co., Ltd., to acquire raw data.

1.6 Processing of sequence data

The raw reads were filtered using the software of FLASH by removing raw reads containing more than 10% of unknown nucleotides (N) and less than 80% of bases with quality (Q-value)>20. Paired-end clean reads were merged as raw tags a minimum overlap of 10 bp, and remove the merged reads that the mismatch ratio in overlapping regions larger than 2%[28]. By this method, we obtained effective tags for further analysis.

The effective tags were clustered into operational taxonomic units (OTUs) by greengene database (version 20 101 006) with Confidence Threshold of 0.5 using the software rdp classifier (http://rdp.cme.msu.edu/classifier/classifier.jsp)[29]. The effective tags were clustered into the same OTUs of ≥97% similarity using UPARSE pipeline[30]. The abundance of each sample on taxonomic level of domain, phylum, class, order, family, genus and species were calculated according to the taxonomic classification and abundance of OTU. All the analyses from clustering to alpha (within sample) and beta diversity (between samples) was performed with

QIIME program[31]. The characterization of microbiota features was performed by linear discriminant analysis (LDA) effect size (LEfSe) method (http://huttenhower.sph. harvard.edu/lefse/).

1.7 Statistical analysis

Statistical differences were determined by using SPSS software (version 25.0). Numerical data was presented as the mean±standard error, and between-treatment differences were analyzed with a one-way analysis of variance (ANOVA). The threshold for statistical significance was set at $P<0.05$.

2 Results and analysis

2.1 Changes in water quality during culture and the growth of shrimp

At the end of the five-week culture experiment, the concentration of ammonium and nitrite in the culture water was measured (Table 1). The results showed that there was no significant difference in the concentration of ammonium or nitrite among the three groups. The growth indicators of the three groups of shrimps are shown in Table 2. The survival ratio (SR) and specific growth rate (SGR) of shrimp showed no significant difference between the control and *Bacillus licheniformis*-treated group.

2.2 Characteristics of 16S rRNA gene sequencing

To determine the microbiota between *P. vannamei* intestine, water and sediment, bacterial 16S rRNA gene V3–V4 regions were conducted (Table 3). In this study, a total tags number of 2 474 025 of V3–V4 denoised 16S rRNA gene reads were obtained from 27 samples, with an average of 91 630 tags for each sample (the minimum of one sample was 85 138 and the maximum was 98 952). The rarefaction curves of the samples tended to approach the saturation plateau, indicating that the gene sequence database was abundant and enough for the microbial diversity analysis. Then these sequences were classified into the same OTUs (operational taxonomic units) at an identity threshold of 97% similarity by using Mothur (v.1.34.0). The total tags, OTUs, statistical estimates of species richness and diversity index from each group were showed in Table 3. The results showed that Chao indexes were different in the IAW group compared with IC group, but not different in the water and sediment groups. Shannon indexes were different in the SC group compared with SAW and SAF group.

Table 1 Effects of different adding methods on the ammonium concentration and nitrite

Treatments	Ammonium concentration (mg/L)	Nitrite concentration (mg/L)
Control	1.4±0.30	0.04±0.05
Add in water	1.6±0.17	0.07±0.03
Add in feed	1.6±0.17	0.04±0.05

Value is the mean±SD of three independent repeats.

Table 2 Effects of different adding methods on the growth of shrimp

Treatments	Control	Add in feed	Add in feed
IBW (g)	0.506±0.142	0.584±0.024	0.528±0.175
FBW (g)	3.201±0.236	2.921±0.524	3.208±0.006
SGR (%/d)	0.050±0.002	0.046±0.004	0.053±0.009
SR (%)	89±2.1	94±9.2	87±0.1

Value is the mean±SD of three independent repeats. IBW: Initial body weight; FBW: Final body weight; SGR: Specific growth rate; SR: Survival rate.

Table 3 Richness and diversity indexes relative to each sample (OTU cutoff of 0.03)

Sample	Groups	Total tags	OTUs	Chao	Ace	Shannon	Simpson
Intestine	IC	89 953±4 872	699±34a	992.64±31.59a	1 018.23±23.83	5.05±0.56	0.90±0.04a
	IAW	91 184±1 250	777±29b	1 094.18±38.23b	1 098.04±79.62	5.64±0.19	0.96±0.01b
	IAF	94 316±5 280	741±29ab	1 035.02±64.39ab	1 052.87±81.89	5.43±0.21	0.93±0.02ab
Water	WC	93 023±5 271	741±53	1 065.52±167.02	1 038.02±140.97	6.19±0.40	0.96±0.02
	WAW	92 443±3 531	748±45	1 063.12±41.70	1 024.40±40.95	6.12±0.09	0.97±0.00
	WAF	92 035±3 817	689±85	1 031.26±103.09	1 022.56±46.20	5.88±0.07	0.96±0.00
Sediment	SC	89 272±2 466a	867±26	1 225.12±51.25	1 182.17±27.23	6.50±0.10b	0.97±0.01b
	SAW	94 528±2 383b	860±42	1 232.31±111.18	1 219.31±104.55	5.62±0.10a	0.94±0.00a
	SAF	87 921±2 539a	833±56	1 150.53±108.26	1 167.21±77.30	5.32±0.50a	0.92±0.02a

Value is the mean±SD of three independent repeats. Different letters (a, b) represent a significant difference within groups (one-way ANOVA).

2.3 Overall microbiota structures

OTUs were identified into 23, 20 and 27 prokaryotic phyla from the 16S rRNA gene sequences in intestine, water and sediment. The relative abundant phyla in all samples were *Planctomycetes* (36%), *Proteobacteria* (30%), *Verrucomicrobia* (9%), *Actinobacteria* (8%), *Bacteroidetes* (7%), *Patescibacteria* (5%), *Chloroflexi* (2%), *Chlamydiales* (1%), and others (2%) (Figure 1). The species composition of the intestine was very similar to that of the sediment, with *Planctomycetes*, *Proteobacteria*, *Verrucomicrobia* accounting for the top three species, water also has the above species, but the proportion is different.

In order to explore the effect of probiotics addition on microbial community, we analyzed OTU of different treatment groups in intestine, water and sediment, Veen diagram was constructed to identify dominant OTUs presented in these three groups (Figure 2). The Venn diagram showed that there were 322 common OTUs in the 9 groups of intestines, representing 31% of the total reads (Figure 2A). And there were 374 OTUs shared among 9 samples in water, representing 35% of the total reads (Figure 2B). There were 398 OTUs shared among SC, SAW and SAF, representing 34% of the total reads in sediment (Figure 2C). The Venn diagram also showed that in the intestinal

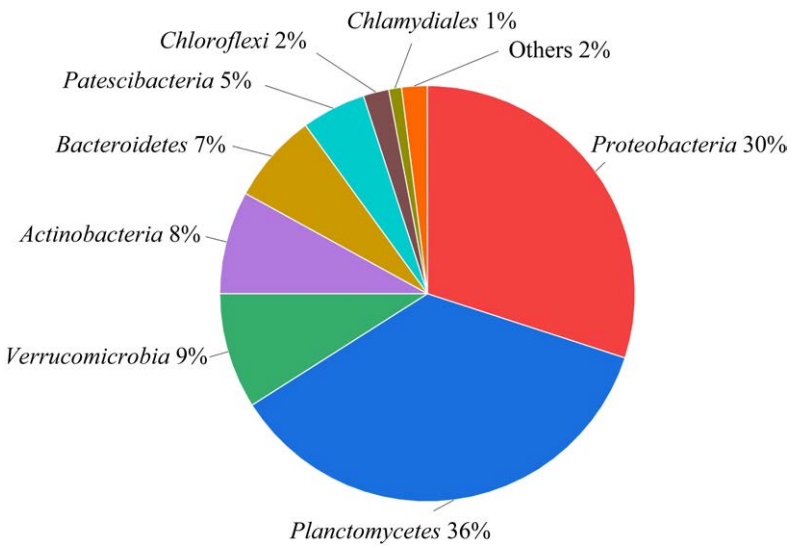

Figure 1 The bacterial community in all samples at phylum level.

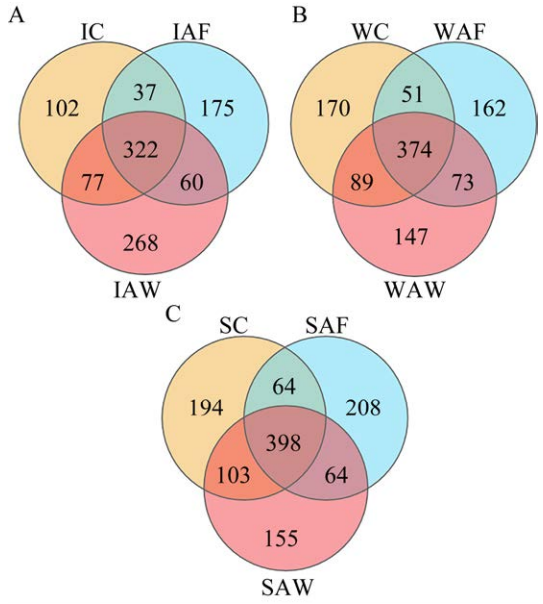

Figure 2 Comparison of OTUs in intestine (A), water (B), and sediment (C) by Venn diagram.

samples, the number of OTU specific to each group was significantly different. Moreover, different ways of addition have different effects on the specific OTU level. This was not found in water bodies and sediments.

At the same time, the OTU of the intestine and environment in different treatment groups was studied (Figure 3). In the control group, there were 362 common OTUs in the 9 groups of intestine, water and sediment, representing 34.51% of the total reads (Figure 3A). In the AW group, there were 355 common OTUs in the 9 groups of intestine, water and sediment, representing 28.56% of the total reads (Figure 3B). There were 325 OTUs shared among intestine, water and sediment, representing 27.61% of the total reads in AF group (Figure 3C).

2.4 Bacterial composition and community structure

The bacterial composition of intestine, water and sediment at different samples at phylum and genus level were show in Figure 4A. The dominant phyla in the nine groups were *Planctomycetes*, *Firmicutes*, *Chloroflexi*, *Chlamydiae*, *Verrucomicrobia*, *Proteobacteria*, *Actinobacteria*, *Patescibacteria*, *Bacteroidetes*, and *Acidobacteria*.

In the intestine groups, the dominant phyla were *Planctomycetes*. The abundance of *Chlamydiae* was increased in the IAF group, and decreased in the IAW group compared with the control group (P<0.05). And in both treatment groups that added probiotics, there was decrease in *Verrucomicrobia* abundance, although not statistically significant. The dominant phyla in the water groups were *Proteobacteria*. Figure 4A also demonstrated *Chlamydiae* had high abundance in the WAF group, and decreased in the WAW group compared with the control group (P<0.05). In the sediment groups, the dominant phyla were *Planctomycetes* and *Proteobacteria*. The abundance of *Planctomycetes* was increased in the SAW group, and decreased in the SAF group compared with the control group (P<0.05). These results indicate that there are some differences in microbial community between the intestine and environment. The abundance of *Chlamydiae* was increased in the group that probiotic was added feed, and decreased in the group that probiotic was added water, compared with the control group. Both the intestine and the water samples showed similar trends.

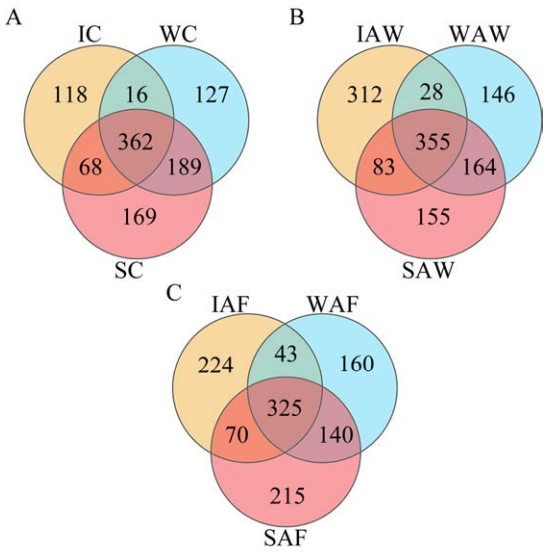

Figure 3 Comparison of OTUs in control group (A), AW group (B), and AF group (C) by Venn diagram.

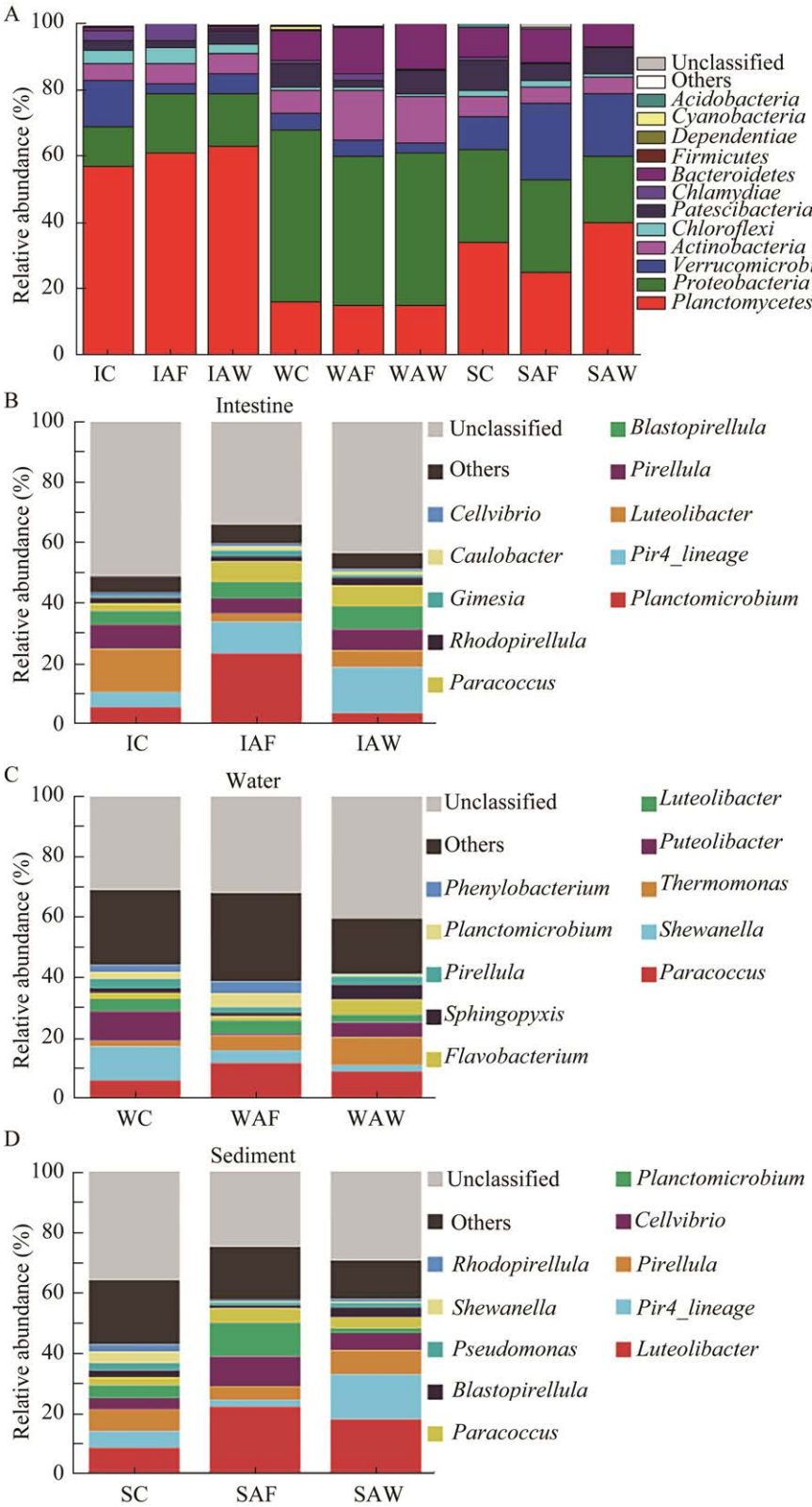

Figure 4 Microbiota composition of bacterial taxa at phylum (A) and genus for intestine (B), water (C), and sediment (D).

From the Figure 4B–4D, we can see that in the intestine, water or sediment samples, the composition of the dominant genus of each experimental group is different from the control group. Similarly, the dominant genus of group of the probiotics added to the feed and the water is also relatively different. In the intestine samples, the abundance of *Planctomicrobium* was significantly decreased in the IAW group, and increased in the IAF group (P<0.05), while the abundance of Pir4_lineage was significantly increased in the IAW group and IAF group (P<0.05). There is a similar trend of the abundance of *Planctomicrobium* in the water samples. In the sediment samples, the abundance of Pir4_lineage was significantly decreased in the SAF group, and increased in the SAW group (P<0.05). The results showed that adding probiotics to water and feed had similar changes in intestine and water microbial communities at genus level.

Additionally, samples of the intestine, water and sediment tended to cluster together by principal coordinates analysis (PCoA) analysis (Figure 5). In intestine and environmental samples, the three repeated samples in each treatment group were close to each other, indicating that the samples were similar (Figure 5A–5C). And each treatment group shows the phenomenon of individual aggregation. At the same time, we also explored the relationship between the microbial communities in the intestines, water and sediments in different treatment groups. Additionally, samples of the intestine, water and sediment at the control group tended to cluster together by PCoA analysis with PC1=73.40% and PC2=15.21% of total variations (Figure 5D), and the distance between the different samples is relatively close, which further confirms the similarity between the bacterial community in the shrimp intestine and the surrounding environment. In the two groups with probiotics added (Figure 5E, 5F), it was found that the distance between different samples was relatively long, indicating that the bacterial community structure in the intestinal, water and sediment samples was quite different.

2.5 Functional prediction of the microbiota

The presumptive functions of the microbiota of intestine, water and sediment were illustrated using PICRUST. The Kyoto encyclopedia of genes and genomes (KEGG) pathway analysis demonstrated the top 20 pathways for each group (Figure 6A). And those pathways associated with

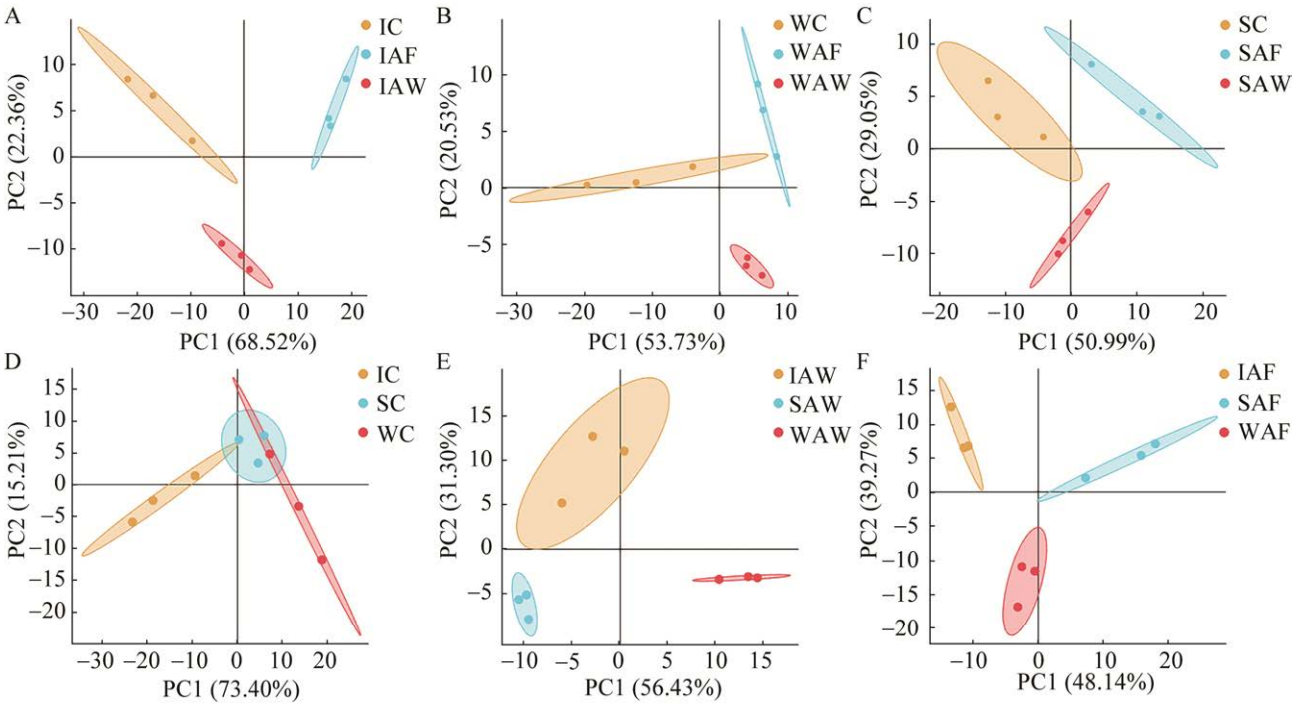

Figure 5 Principal coordinates analysis (PCoA) plots based on different samples (A, B, C) and groups (D, E, F). A–C stands for intestine, water, and sediment, respectively. D–F stands for control group, AW group, and AF group, respectively.

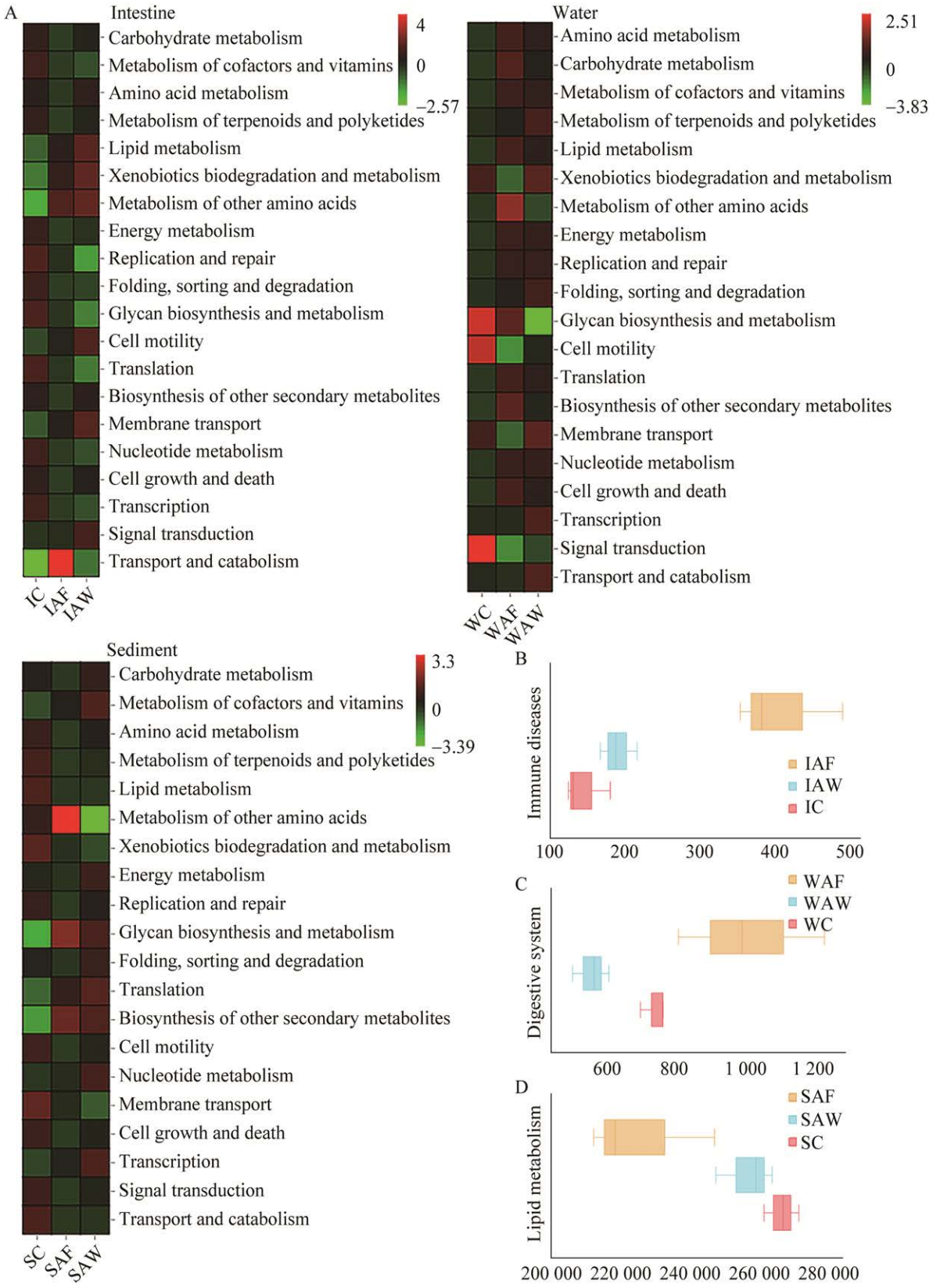

Figure 6 Heat map of the presentation of KEGG of the pathways in the 12 samples (A), and comparison of altered pathways in intestine (B), water (C) and sediment (D).

cellular processes (cell motility, cell growth and death), genetic information processing (e.g., transport and catabolism, transcription), and metabolism (e.g., amino acid metabolism, carbohydrate metabolism, xenobiotics biodegradation and metabolism, glycan biosynthesis and metabolism, etc.). Welch's *t*-test results (Figure 6B–6D) indicated that several predicted pathways were significantly enriched (95% confidence intervals, $P<0.05$) in some samples. In the shrimp intestine, the immune system was up-regulated in IAW and IAF (Figure 6B). In the water, the digestive system was up-regulated in WAF, but down-regulated in WAW (Figure 6C). In the sediment, the lipid metabolism system was down-regulated in the SAW and SAF (Figure 6D). The data showed that added probiotics enhanced the immune pathways in the shrimp intestine, whereas in the environment, the digestive system and lipid metabolism system were affected.

3 Discussion

The shrimp farming has developed rapidly in recent years[32-33], but some diseases of the shrimp, such as acute hepatopancreatic necrosis syndrome (AHPNS)[34] or early mortality syndrome (EMS)[35], still plague the farmers. Faced with this problem, probiotics are considered a good choice. And among them, *Bacillus licheniformis* has been widely used in aquaculture[22,36]. A present report showed that *Bacillus licheniformis* can resistance pathogenic microorganisms, and reduce the occurrence of diseases[37]; enhance the immune function of the animal body[38]; produce a variety of digestive enzymes, increase daily gain and feed utilization, and reduce production costs[16,39]. However, there are few studies on the role of *Bacillus licheniformis* in regulating the balance of intestinal flora and its surrounding environment of shrimp[19,40-41]. In order to study this problem more accurately, we have chosen the zero-water exchange aquaculture system, which can reduce the impact of external factors on the aquaculture system[42-43].

In aquaculture, *Chlamydia* is a common pathogen in fish, and *Chlamydia* infection is becoming an important pathogenic cause in aquaculture[44-45]. Studies show that fish on the common skin inflammation diseases are caused by *Chlamydia*, this type of parasitic pathogens in fish gills, fins, and body surface skin cells, form large inclusion body in cell proliferation after, cause cyst cause epithelial cell hypertrophy, nearly caused by fish breathing difficulties, metabolic disorders, the fatality rate[46-47]. A previous report showed that the proportion of *Chlamydia* in water increased significantly with the passage of culture time, which was a hidden danger of shrimp disease[48-49]. In this study, the proportion of *Chlamydia* added by *Bacillus licheniformis* to the water group decreased significantly, which played an important role in reducing the incidence of *P. vannamei* in the zero-water exchange aquaculture system. This helps us to reasonably choose the method of adding bacteria according to the needs of breeding, in order to achieve the purpose of saving costs.

Firmicutes in the intestinal tract of obese people has a significant upward trend than that of the normal population. When the obese people lose weight, the number of *Firmicutes* in the intestinal tract returns to the level of the normal population[50]. In the study of the intestinal flora of the transgenic carp and wild carp, studies have shown that with the expression of the transgenic carp, the level of *Firmicutes* in the intestinal tract of the transgenic carp has an upward trend[51]. In this experiment, in the two groups added with *Bacillus licheniformis*, the proportion of *Firmicutes* increased, indicating that the addition of *Bacillus licheniformis* will increase the number of *Firmicutes*, which may be *Bacillus licheniformis* promote the growth of animals the reason.

Existing research shows that probiotics may play a role by competing with certain pathogenic bacteria for adhesion sites and nutrients[45,48-49,52]. Therefore, studying the microbial communities change in intestines and environment after the

addition of probiotics plays an important role in understanding the mechanism of probiotics[50]. In this study, we found that the addition of *Bacillus licheniformis* had a greater impact on the abundance at the level of the bacterial phyla. Among them, *Chlamydia* and *Firmicutes* had a significant impact. In the AF group, the abundance of *Chlamydia* in the intestine and water was increased; the trend of *Chlamydia* in the AW group was just the opposite. In addition, in the sediment samples, the *Planctomycete* phylum was significantly down-regulated in the AF group, while the opposite was true in the AW group. This suggests that when *Bacillus licheniformis* is added to feed or water, it may play a role by changing the abundance of *Chlamydia*[53-54].

Planctomycetes sp. plays key roles in biogeochemical transformations of carbon and nitrogen cycles because there are specialized for the initial breakdown of various highly complex polysaccharides[55]. Previous studies have shown that Pir4_lineage belongs to *Planctomycetes*[40,56]. In this study, whether it is intestine or water and sediment, the genus with significant differences mainly concentrated in *Planctomicrobium* and *Pir4_lineage*. *Planctomicrobium* has a consistent trend in the intestine and water, both of which AF increase and AW group decrease. However, Pir4_lineage increased in both the AF and AW groups in the intestine. And in sediment, Pir4_lineage increased in SAW and decreased in SAF. These results revealed that the addition of *Bacillus licheniformis* may change the decomposition of organic matter by shrimps. We also speculate that *Bacillus licheniformis* may have a synergistic effect with Pir4_lineage. In some studies, *Planctomicrobium* and Pir4_lineage have been proved to be harmless to the environment[57], so this can also show that the water and sediment after adding probiotics will not change adversely to the environment, which can prevent damage to the surrounding environment.

KEGG results showed that the dominating functional categories in three groups were mostly related to metabolism and genetic information process, such as amino acid metabolism, carbohydrate metabolism, energy metabolism, replication and repair, and membrane transport. It is basically similar to the conclusions of previous studies on shrimp intestine and living environment microorganisms[58-59], indicating that amino acids[60] and carbohydrates may be one of the main nutrients digested by intestinal microbiota. Additionally, "immune diseases" was enriched in probiotic-add group in intestine, this indicating that the addition of probiotics may increase the immunity of the intestinal flora, and thus improve the ability to resist pathogens. This may be the microbiological reason why some studies have found that the addition of probiotics will increase the expression of immune genes in shrimp[61-63]. Likewise, the relative abundance of "lipid metabolism" in control group was different from the probiotic-add group in sediment. And, it is possible that the added-probiotics group produced less lipid in the sediment, which may be related to the previous study that the probiotics added can increase the absorption of nutrients in the intestine[19,64-65].

In this study, we tried to validate the role of *Bacillus licheniformis* in shrimp culture by intestinal and surrounding bacterial communities. The results showed that under normal conditions, there are similarities in the bacterial communities of the shrimp intestines and the surrounding environment in the zero-water exchange aquaculture system. The addition of *Bacillus licheniformis* will greatly change the species richness at the phylum and genus level. From the functional analysis, we found that the addition of probiotics to feed and water changed the related pathways, for example, digestive system, but the change trend was opposite. And adding probiotics in different ways has different effects on the microbial community of intestine, water and sediment. This study provided an idea for how to effectively select the addition method of *Bacillus licheniformis* to improve the intestinal health of shrimps and promote the environmentally friendly development.

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