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Bacterial Population in Intestines of *Litopenaeus vannamei* Fed Different Probiotics or Probiotic Supernatant

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The interactions of microbiota in the gut play an important role in promoting or maintaining the health of hosts. In this study, in order to investigate and compare the effects of dietary supplementation with *Lactobacillus pentosus* HC-2 (HC-2), *Enterococcus faecium* NRW-2, or the bacteria-free supernatant of a HC-2 culture on the bacterial composition of *Litopenaeus vannamei*, Illumina sequencing of the V1-V2 region of the 16S rRNA gene was used. The results showed that unique species exclusively existed in specific dietary groups, and the abundance of Actinobacteria was significantly increased in the intestinal bacterial community of shrimp fed with the bacteria-free supernatant of an HC-2 culture compared with the control. In addition, the histology of intestines of the shrimp from the four dietary groups was also described, but no obvious improvements in the intestinal histology were observed. The findings in this work will help to promote the understanding of the roles of intestinal bacteria in shrimps when fed with probiotics or probiotic supernatant.

Keywords: Probiotics, supernatant, bacterial composition, histology, *Litopenaeus vannamei*

Introduction

Shrimps are a major marine product, with high economic value, but their commercial production declines sharply with reductions in water quality, increased stress, and bacterial or viral infections [37]. In most cases, bacterial diseases can be prevented by the appropriate management of shrimp culture [9], by maintaining balanced microbial communities in shrimp farms that include pathogenic, innocuous, and beneficial bacteria. The bacterial communities in the nearby marine environment affect the intestinal bacterial microbiota of farmed marine animals [11]. Some bacteria in the intestine can be pathogenic to their host, whereas others are beneficial to the development, morphology, and physiology of the host, contributing to nutrient absorption, immune responses, and epithelial development [13, 28].

Probiotics, as one alternative to antibiotics, have been commonly administered in aquaculture because they confer several benefits, including immunological, nutritional, and environmental benefits [20, 33]. Among the probiotics available, some kinds of lactic acid bacteria are widely used because they play important roles in the host digestive tract, improving its immune status, modulating the bacterial community, and antagonizing opportunistic pathogens [16, 25, 37]. In our previous study, *Lactobacillus pentosus* HC-2 (HC-2) and *Enterococcus faecium* NRW-2 (NRW-2) were isolated from aquatic animals (*Acanthogobius hasta*) based on their antibacterial and adhesive activities, respectively, and were shown to enhance the immune responses and disease resistance of shrimp compared with those of the controls. In particular, dietary supplementation with strain HC-2 significantly promoted the growth performance of shrimp relative to that of the control group [31]. To advance the utilization of these probiotics, the mechanism of their probiotic action must be investigated. However, studies of their modes of action in the penaeids have focused on their direct inhibition of pathogens and their immunomodulatory effects, which are just two of the many mechanisms of probiotic action [30]. Our knowledge of the effects of

probiotics on the gut microbiota of invertebrates, including *Litopenaeus vannamei* [24], Kuruma shrimp *Marsupenaeus japonicus* [22], and Chinese fleshy prawn *Fenneropenaeus chinensis* [23], is limited. Therefore, to understand the relationships between the intestinal microbiota and the health of these microbes, and to evaluate the effects of different diets on the shrimp microbiota and intestine, a fundamental knowledge of these intestinal microbial populations and their effects on the shrimp intestinal morphology is required. In this study, we investigated the intestinal histology and bacterial composition of the gut microbiota in shrimp fed with *E. faecium* NRW-2 and *L. pentosus* HC-2, and shrimp fed with the corresponding supernatant of *L. pentosus* HC-2 because of the high antibacterial activity of *L. pentosus* HC-2.

Materials and Methods

Strains

Lactobacillus pentosus HC-2 (HC-2) (GenBank Accession No. KU995298) and *Enterococcus faecium* NRW-2 (NRW-2) (GenBank Accession No. KU995299) were used in this study, and were isolated from the gut of a healthy fish (*Acanthogobius hasta*) based on their antibacterial and adhesive activities, respectively (the details of source and identification of bacteria can be found in Sha *et al*. [31]). For the experiments, the bacterial strains were cultured in the Man-Rogosa-Sharpe broth for 24 h at 37°C. The cells were harvested after the cultures were centrifuged (13,000 ×*g*, 4°C, 10 min) and washed with sterilized seawater. The supernatants were filtered (0.22 μm) and the cells were resuspended in sterilized seawater before use.

Preparation of Experimental Diets

The experimental diets were generated as described previously by Sha *et al*. [31]. In brief, cells were resuspended in sterilized seawater and sprayed on basal feed at 1×10^7 colony-forming units (CFU) g/feed, as well as the corresponding filtered (0.22 μm) supernatant of HC-2. The feed was dried at room temperature for 5 h and stored at 4°C, and the cell viability was evaluated every day after preparation. We found that the cell viability at day 8 was still close to the initial level of CFU added to the feed, so the feed would be prepared every 7 days.

Feeding Experiment

To analyze the effects of strains HC-2 and NRW-2 and the supernatant of HC-2 on the intestinal bacterial diversity and intestinal morphology of the shrimp, experiments were designed as follows: H, shrimp fed a basal diet + strain HC-2 (1×10^7 CFU/g feed); N, shrimp fed a basal diet + strain NRW-2 (1×10^7 CFU/g feed); S, shrimp fed a basal diet + strain HC-2 bacteria-free supernatant (the amount of supernatant corresponding to that of 1×10^7 CFU/g feed of HC-2); and C, shrimp fed a basal diet alone as the control. The basal diet was obtained from Yantai Dale Feed Co., Ltd (China), containing crude protein 42%, crude fat 7%, ash 15%, and water 11%. Triplicate samples were set up per treatment. Conditions of the feeding experiment were the same with our previous study [31]. In brief, all shrimps were maintained in fresh seawater (salinity, 30‰) at 30 ± 2°C with continuous aeration and a 60% water change every day. The daily feeding rate was 10% of the body weight. Animals were fed three times per day at 7:00, 11:00, and 19:00, and the respective feeding rates were 35%, 20%, and 45%, accounting for 1 day's feeding rate. Uneaten feed and feces were removed every day [31].

Sample Collection and Sequencing

After the 4-week feeding experiment, the shrimps were starved for 1 day, and the guts of three shrimps from each tank were pooled into one sample, with triplicate samples per treatment. These samples were saved in ice and sent to Shanghai Personal Biotechnology Co., Ltd for DNA extraction and Illumina sequencing.

Sequence Analysis

Polymerase chain reaction products were generated with the primer pair 8F (5′-AGAGATTTGATCCTGGCTCAG-3′) and 338R (5′-TGCTGCCTCCCGTAGGAGT-3′), which amplified the V1 and V2 regions of the 16S rRNA gene. The products were sequenced with the Illumina Miseq platform. The short reads that overlapped were assembled with FLASH [8] and the low-quality assembled reads were filtered with QIIME [34]. In QIIME, poor-quality sequences were set as sequences with a length less than 150 bp, and contained ambiguous bases. All the sequences were classified from phylum to species levels based on the Greengene [5] and taxonomic trees were constructed with MEGAN [15] using taxonomic assignments. To obtain more information about the bacterial diversity in our samples, the reads acquired were clustered with the QIIME software based on a similarity of at least 97% 16S rRNA sequence self-similarities rather than by matching them to an external database, and were classified into operational taxonomic units (OTUs). Chao biodiversity index, ACE index, Shannon index, and Simpson index values were calculated for each shrimp library using MOTHUR.

A beta diversity analysis was used to compare the microbial community compositions in the different samples with QIIME [34], including weighted and unweighted UniFrac. A principal components analysis (PCA) and a principal coordinates analysis (PCoA) were conducted based on the unweighted UniFrac distance.

Histology of the Midgut

Upon termination of the experiment, the midguts of three shrimps from each treatment group were dissected and fixed (60% absolute ethanol, 30% trichloromethane, 10% acetic acid) for 19 h. The fixed tissues were dehydrated in ascending concentrations of alcohol, cleared in toluene, embedded in paraffin, and sectioned at 10 μm with a rotary microtome. The sectioned tissues were stained with hematoxylin and eosin, and images were obtained

with a light microscope as previously described [2].

Statistical Analysis

Data representing the abundances of the OTUs were analyzed with the SPSS software (ver. 17.0). One-way analysis of variance was used to analyze the differences among the different treatment groups. $p < 0.05$ was considered significant.

Data deposition: All sequences have been submitted to the NCBI with the BioProject accession number SRP071046.

Results

Sequences Obtained

To determine the bacterial microbiota, 16S rRNA gene amplicons from the intestinal samples from shrimps of groups H, N, S, and C were sequenced with Illumina. A total of 672,840 sequences of the V1–V2 region of the 16S rRNA gene were obtained with Illumina sequencing and with an average read length of 297 ± 20 bases (Table 1). All sequences were sorted with a barcode for each group: C (181,214 sequences), H (137,385 sequences), N (172,400 sequences), and S (181,841 sequences).

Richness and Diversity

The sequences were clustered into OTUs at a dissimilarity level of 0.03, and each OTU represented a unique phylotype. The total number of OTUs was highest in group H (3,613 OTUs), whereas groups C, N, and S contained 2,923, 3,595, and 2,757 OTUs, respectively (Table 1).

A rarefaction analysis was performed to determine whether adequate OTUs had been obtained from each sample with Illumina sequencing. These curves showed that all the samples did not reach saturation for the four treatment groups even in most of the libraries that contained >70,000 reads, suggesting that more bacterial species are still expected in shrimp guts (Fig. S1), especially in the guts of group S (S1, S2, S3). The community richness (Table 1) was calculated with the Chao/Ace ratio based on the numbers of OTUs at the 0.03 dissimilarity level. The Chao/Ace ratios were higher in groups H and N than in groups S and C, and was higher in group C than in group S. Among all the samples, the Chao/Ace ratio was highest in group H. These results indicate that the bacterial community richness was higher in group H than in the other three groups, and that the richness in group C was higher than in group S but lower than in group N (H > N > C > S).

Bacterial diversity was estimated with Simpson's and Shannon's indices. The value for Simpson's index was the same in groups H, N, and S, and was higher than in group C (H = N = S > C). However, Shannon's index was higher in group C than in the other three groups, and group S was higher than group N, and group H was the lowest (C > S > N > H).

A Venn diagram (Fig. S2) of the OTUs at the 0.03 dissimilarity level showed that in total, 4,605 species of bacteria were found in all the samples in the four groups, and there were distinct differences among the microbial structures of the four groups. Seventy-six species belonged uniquely to group C, and the only OTUs present in all the subsets of group C belonged to the genus *Sutterella*. Group H had the greatest number of unique species (202 in total) of the four groups, and the OTUs present in all subsets of group H were *Paracoccus* sp., *Polymorphum gilvum*, *Methylobacterium*

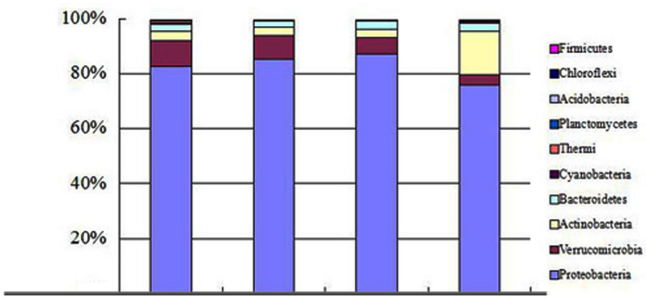
Table 1. The assignment and analysis of sequences.

	Different treatments			
	C	H	N	S
Sampling depth				
A total number of sequences	181,214	137,385	172,400	181,841
OTUs (0.03 dissimilarity level)	2,923	3,613	3,595	2,757
Phylum	26	27	28	29
Class	69	72	72	74
Family	129	136	135	139
Genus	222	234	240	237
Diversity index and estimated OTU richness				
Chao/Ace	0.72 ± 0.03	0.78 ± 0.03	0.76 ± 0.02	0.71 ± 0.05
Simpson	0.10 ± 0.02	0.14 ± 0.02	0.14 ± 0.01	0.14 ± 0.07
Shannon	3.60 ± 0.23	3.27 ± 0.10	3.31 ± 0.11	3.47 ± 0.49

Shrimps were fed a basal diet (C) or a basal diet supplemented with *L. pentosus* HC-2 (H), *E. faecium* NRW-2 (N), or the supernatant of *L. pentosus* HC-2 (S).

sp., and members classified as WPS-2 (phylum) and Rhodobacteraceae (family). One hundred ninety-six species belonged uniquely to group N, and the OTUs present in all the subsets of group N were *Rhodobacter veldkampii* and members classified as *Octadecabacter*, Rhodobacteraceae (family), Alphaproteobacteria (class), *Octadecabacter*, and Kiloniellales (order). Group S had a smaller number of unique species (78 in total) than groups H and N, and the OTUs present in all the subsets of group S were *Methylobacterium* and members of Rhodobacteraceae (family). The relative abundance of OTUs uniquely present in all the subsets of each group was about 0.1%.

Based on a BLAST analysis, the OTUs shared by the four groups were classified to the nearest taxonomic ranking to determine the common species, which included Actinomycetales, Flavobacteria, Alphaproteobacteria, Betaproteobacteria, Gammaproteobacteria, and Verrucomicrobiae. Class Alphaproteobacteria contained the most shared OTUs among these 12 specimens, including Caulobacteraceae, Brucellaceae, and Rhodobacteraceae. Members of the family Rhodobacteraceae were the dominant species shared in all the samples (Table S1), although the number of reads varied in different individuals, suggesting that this family is part of the core intestinal bacteria of *L. vannamei*. Dietary supplementation with probiotics or supernatant did not produce uniquely shared species in the intestines of all the subsets of shrimp from groups H, N, or S compared with the control, but altered the richness of some species (data not shown). Interestingly, the addition of HC-2 bacteria-free supernatant reduced the species in the gut of the shrimp, including *Maribius salinus*, *Haloferula phyci*, *Phaeobacter gallaeciensis*, and *Octadecabacter* sp.



Phylum	C (%)	H (%)	N (%)	S (%)
Proteobacteria	82.52±4.69	85.13±1.07	87.10±1.56	75.31±14.57
Verrucomicrobia	9.11±5.99	8.57±1.33	5.79±1.07	3.78±1.009
Actinobacteria	3.44[a]±0.70	2.85[a]±0.59	2.94[a]±0.11	15.92[b]±11.41
Bacteroidetes	2.48±0.28	2.41±0.65	3.11±1.03	3.03±1.43
Cyanobacteria	0.47±0.29	0.32±0.03	0.36±0.08	0.59±0.36
Thermi	0.90±1.35	0.10±0.02	0.15±0.07	0.25±0.08
Planctomycetes	0.42±0.31	0.22±0.07	0.12±0.02	0.23±0.09
Acidobacteria	0.10±0.06	0.15±0.13	0.11±0.10	0.17±0.09
Chloroflexi	0.19±0.26	0.07±0.04	0.07±0.04	0.19±0.07
Firmicutes	0.10±0.07	0.04±0.00	0.07±0.03	0.09±0.07

Fig. 1. Relative abundance of the top 10 phyla in the four treatment groups.
Shrimps were fed a basal diet (C) or a basal diet supplemented with *L. pentosus* HC-2 (H), *E. faecium* NRW-2 (N), or the supernatant of *L. pentosus* HC-2 (S).

Bacterial Composition and Community Structure

Based on the RDP classifier, the smallest number of bacteria phyla was detected in samples from group C, whereas the samples from group S had the highest number of bacterial phyla (26, 27, 28, and 29 phyla for groups C, H, N, and S, respectively). The top 10 phyla and their relative abundance are shown in Fig. 1. Proteobacteria were the

Table 2. Top 10 genera and their relative abundance.

Genus	Different treatments			
	C (%)	H (%)	N (%)	S (%)
Octadecabacter	2.70 ± 0.67[a]	2.70 ± 0.92[a]	3.89 ± 0.35[b]	1.57 ± 0.22[ac]
Acinetobacter	1.98 ± 1.48	1.10 ± 0.18	1.16 ± 0.68	2.81 ± 1.81
Demequina	1.09 ± 0.71	0.72 ± 0.28	1.21 ± 0.53	0.71 ± 1.55
Phaeobacter	0.68 ± 0.21[ac]	0.95 ± 0.20[a]	1.04 ± 0.14[b]	0.57 ± 0.07[c]
Haloferula	0.81 ± 0.36[ab]	1.05 ± 0.31[a]	0.82 ± 0.14[ab]	0.43 ± 0.15[b]
Pseudoalteromonas	0.72 ± 0.11	0.50 ± 0.29	1.01 ± 0.57	0.75 ± 0.44
Phenylobacterium	0.58 ± 0.46[ab]	0.36 ± 0.06[a]	0.49 ± 0.14[ab]	1.26 ± 0.67[b]
Paracoccus	0.64 ± 0.24[a]	0.50 ± 0.23[ab]	0.29 ± 0.05[b]	0.34 ± 0.06[ab]
Ochrobactrum	0.36 ± 0.18[ab]	0.26 ± 0.09[b]	0.39 ± 0.23[ab]	0.65 ± 0.19[a]
Ruegeria	0.41 ± 0.15	0.39 ± 0.07	0.39 ± 0.04	0.27 ± 0.12

Values in the same row with different letters differ significantly ($p < 0.05$). Shrimps were fed a basal diet (C) or a basal diet supplemented with *L. pentosus* HC-2 (H), *E. faecium* NRW-2 (N), or the supernatant of *L. pentosus* HC-2 (S).

predominant microflora in the four groups at the phylum level, accounting for more than 75% of all the bacterial communities, followed by Verrucomicrobia, Actinobacteria, Bacteroidetes, Cyanobacteria, Thermi, Planctomycetes, Acidobacteria, Chloroflexi, and Firmicutes. There were no significant differences in the relative abundances of phyla in the four groups, except Actinobacteria ($p < 0.05$). The relative abundance of Actinobacteria was highest in group S, but did not differ significantly among the other three groups.

The top 10 genera and their relative abundance are listed in Table 2. *Octadecabacter* was the predominant genus in groups C, H, and N, and its relative abundance was significantly higher in group N than in group S. However, *Acinetobacter* was predominant in group S, followed by *Octadecabacter* and *Phenylobacterium*, but they did not differ significantly in their abundance among the four groups. The abundance of *Phaeobacter* was significantly higher in group N and lower in group S than in group C. Interestingly, the relative abundances of *Haloferula*, *Phenylobacterium*, and *Ochrobactrum* differed significantly in groups H and S, but not significantly in the other groups, among which only *Haloferula* was higher in group H. The abundance of *Paracoccus* was markedly lower in group N than in group C.

Relationships among the Bacterial Communities in the Four Groups

A heat map was constructed at the genus level based on the bacterial communities across all 12 samples. The analysis showed that the samples generally segregated into two groups (Fig. 2): one group was mainly composed of subgroups S2 and S3, and the other group was composed mainly of samples in groups C, H, and N. A PCoA was performed to determine the relationships among the groups (Fig. 3). Samples of group S were clustered on the left-hand side of the graph, along the first principle component axis (PC1), whereas samples of group C clustered on the right-hand side of the graph. However, samples of group N and H did not cluster well on any side of the graph or along any principle components. The results of the heat map and PCoA were consistent to some extent, indicating that the bacterial communities in groups C, H, N, and S differed and that the bacterial community in the midgut was influenced by the addition of probiotics or probiotic supernatant. The significantly different phyla and genera listed in Table S2 confirm this.

Histology of the Midgut

To investigate the effects of dietary probiotics or probiotic supernatant on the midgut of the shrimp, a histological study was performed at the end of the feeding experiment. The following four images were arbitrarily selected from four groups and represented the intestinal histology of *L. vannamei* in each group. As shown in Fig. S3A, the mucosae shed and piled in the intestinal lumen, and the lamina propria exposed and appeared loose; the apical brush border in some samples appeared normal, with no signs of necrotic enterocytes or cell damage (Figs. S3B and S3D); in the same samples, the mucosae also shed from the intestine but was more serious than that in Fig. S3A (Fig. S3C). However, some individuals showed some reduced folding of the digestive epithelium, which was observed in low-magnification images. This appeared to be the result of individual variations rather than variations among treatments.

Discussion

Previous studies have shown that intestinal bacteria have an important influence on their vertebrate hosts, including in nutrient absorption, immune responses, and modification of the mucosal and gut morphology [10, 14]. Recently, increasing attention has been paid to the relationship between the intestinal microbiota and invertebrate hosts [18, 39]. The factors affecting the intestinal bacteria of different invertebrates have been clarified, including the genetic background, diet, and environment of the host [38], but the interactive influences between the shrimp and its intestinal microbiota and the potential effects of diet on the intestinal microbiota are poorly understood. In our previous study, we had investigated the effects of probiotics and the corresponding supernatant on the growth and immune response of *L. vannamei* [31]; therefore, in this study, the bacterial composition and histology of the midgut of *L. vannamei* fed with different probiotics or bacteria-free probiotic supernatant were characterized.

The improvement in growth achieved with dietary probiotic supplementation has previously been attributed to physiological and biological changes in the intestinal environment and morphological changes in the gut epithelium [3, 21], such as an improved intestinal microvillus structure and a greater absorptive surface area [4, 26]. However, in the present study, the changes in the intestinal microvilli and the folding of the digestive epithelium varied between individuals rather than between dietary groups, and no obvious improvement in intestinal histology was observed. These results are similar to the findings of Daniels *et al.* [3], who reported that dietary *Bacillus* spp.

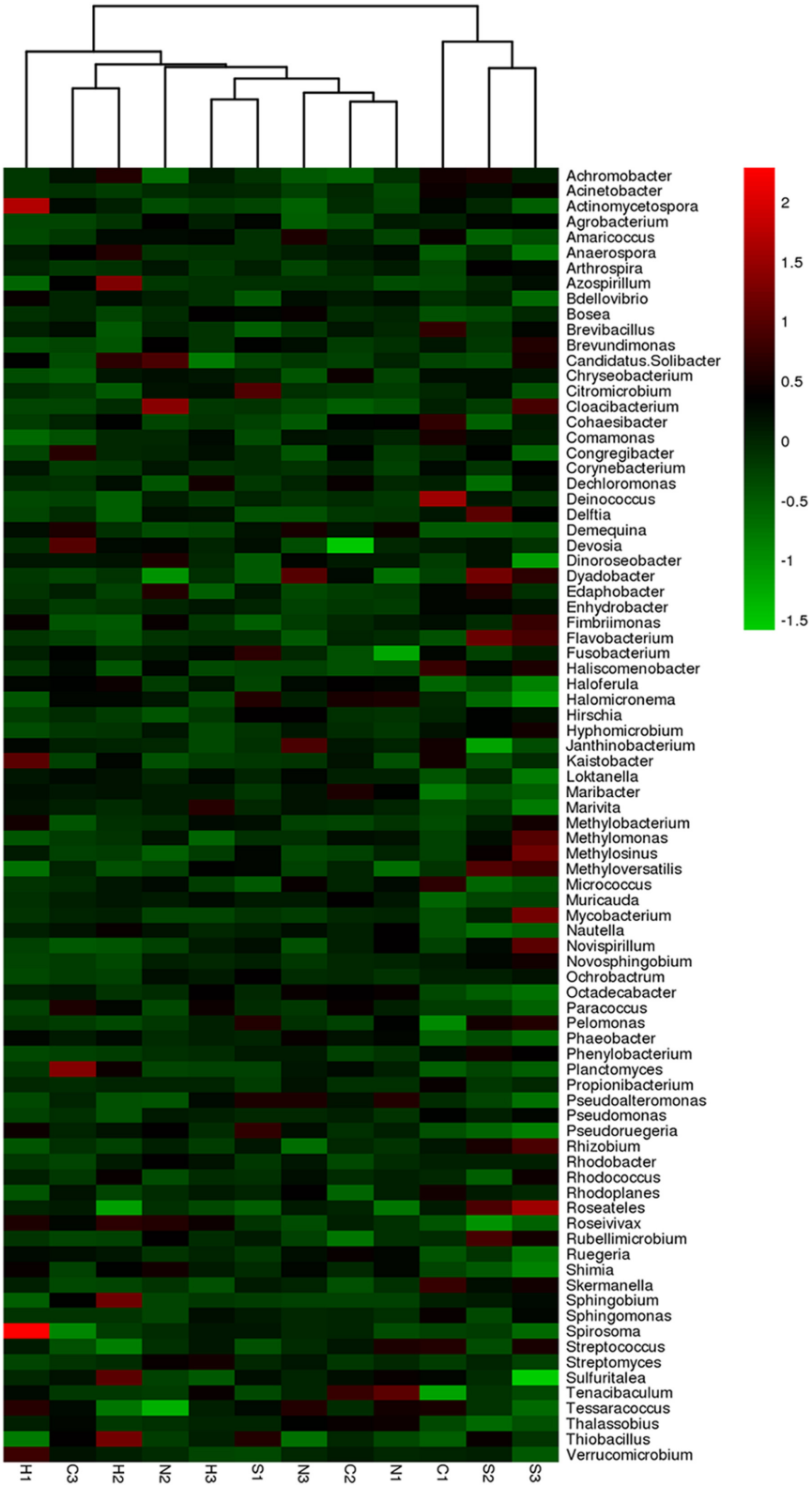


Fig. 2. Heat map of the bacterial diversity in the 12 samples of shrimp.
Shrimps were fed a basal diet (C [C1, C2, C3]) or a basal diet supplemented with *L. pentosus* HC-2 (H [H1, H2, H3]), *E. faecium* NRW-2 (N [N1, N2, N3], or the supernatant of *L. pentosus* HC-2 (S [S1, S2, S3]).

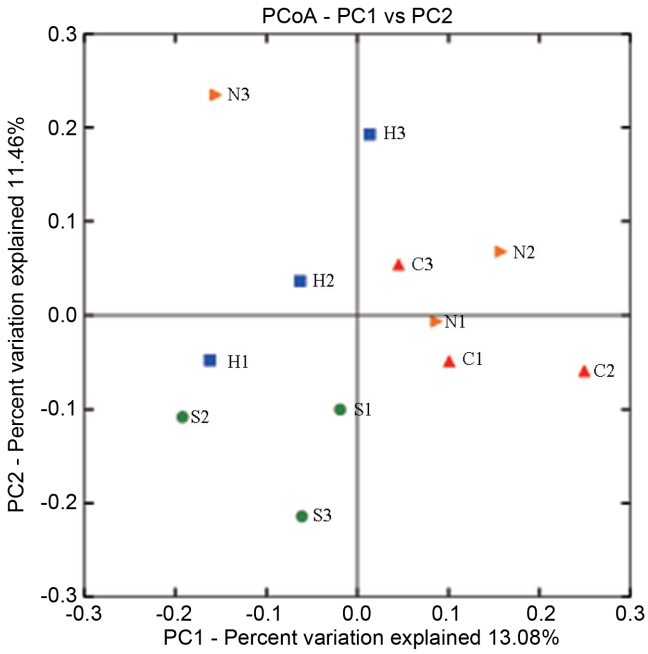


Fig. 3. Principal coordinates analysis scores based on the Unifrac distance.

PC1: the first principle component; PC2: the second principle component. Shrimps were fed a basal diet (C) or a basal diet supplemented with *L. pentosus* HC-2 (H), *E. faecium* NRW-2 (N), or the supernatant of *L. pentosus* HC-2 (S).

and mannan oligosaccharides did not improve the intestinal morphology of *Homarus gammarus*, but are contrary to the findings of Merrifield *et al*. [26], who found that *Pediococcus acidilactici*-fed fish had significantly longer microvilli than other groups of fish. These phenomena may be attributable to the different dietary probiotics given or their styles of action, and these results may suggest that other factors, such as the changes in the intestinal bacterial community, are important in enhancing the growth performance and immune responses of the host organism.

In the present study, Illumina sequencing was used to characterize the bacterial composition in the intestine. Our data showed that each group contained 2,757–3,613 OTUs and 26–29 different phyla, many more than were detected in previous studies [29, 30], in which a sample of wild-caught *Penaeus monodon* had the highest number of OTUs (806) detected with 454 pyrosequencing, as reported by Rungrassamee *et al*. [30]. These discrepancies may result from the different sequencing methods used, and also from the different species of shrimp examined or the culture conditions used. Our Illumina-sequencing data analysis revealed that several OTUs were shared in the *L. vannamei* intestine (Fig. S2, Table S1), among which Rhodobacteraceae (family), belonging to the Proteobacteria (phylum), were dominant in all the samples. Consistent with previous studies, Proteobacteria were the most prevalent members in the shrimps, as in sea cucumbers and fish [11, 29, 35, 39]. Zhang *et al*. [39] reported that Proteobacteria and Tenericutes were the dominant taxa in the intestines of *L. vannamei*, regardless of diet, whereas in this study, the abundance of Tenericutes was too low to determine in all treatment groups, especially group S (data not shown). The discrepancy between this study and Zhang's work may be attributable to the diets or culture conditions used, or to the external environment. It has also been reported that the host intestinal environment exerts a selective pressure on the intestinal microbiota [39]. Strangely, in this study, no *Lactobacillus* and *Enterococcus* were detected. On one hand, it may because their abundance was too low to be detected; on the other hand, it may because of the low adhesion of these strains, which cannot exist in the gut for a long time. However, in this study, the control group was set up and the main goal was to compare the bacterial community between different treatments, so we think that the sequencing data can be used to analyze the effects of dietary supplementation with probiotics and probiotic supernatant on the intestinal microbiota of the shrimp.

In our previous study, dietary supplementation with strain HC-2 significantly enhanced the specific growth rate of the shrimp compared with that of the control group [31]. Surprisingly, dietary supplementation with probiotics did not significantly influence the types of phyla present compared with the control, but altered the types of genera, such as *Paracoccus* sp., *Polymorphum gilvum*, and *Methylobacterium* sp., which were detected only in group H. These may have been associated with the better growth performance in group H than in the other three groups. Members of *Paracoccus* are reported to exert probiotic effects and are used as supplements for some aquatic animals, such as sea cucumbers [36]. *Paracoccus* sp. can also degrade chlorothalonil and biodegrade nitrate [17, 36], so have potential application in improving water quality. In this study, *Paracoccus* species were observed in group C, at higher abundance than in group H, indicating that only some members of the genus *Paracoccus* contribute to the growth of their hosts. *Paracoccus* species were significantly reduced in group N compared with the control, which may confirm indirectly that NRW-2 competes more strongly with *Paracoccus* than HC-2, indicating that NRW-2 and *Paracoccus* share the same or similar adhesive sites in the shrimp intestine. *Polymorphum gilvum* and *Methylobacterium* species are also reported to have oil-degrading and

cypermethrin-degrading capacities [7, 27], respectively, and *Methylobacterium* species have been shown to promote the growth of rice and barley [32], so these species may also exert probiotic effects in *L. vannamei.* In all the treatment groups, only the HC-2 bacteria-free supernatant significantly increased the richness of Actinobacteria and Fibrobacteres compared with the control group, although this varied in the individuals of group S, and contributed to the reduction of some other species, such as *Maribius salinus*, *Haloferula phyci*, *Phaeobacter gallaeciensis*, and *Octadecabacter* sp. This phenomenon is probably attributable to the intestinal environment caused by the HC-2 bacteria-free supernatant, which has a rather low pH (pH ~4), and the members in Actinobacteria and Fibrobacteres may contribute to increase the disease resistance of shrimps [31]. Unfortunately, these changes in the intestinal environment are not well understood and require further study. Dietary supplementation with the supernatant also significantly increased the abundance of many genera (listed in Table S2) compared with the control group, such as *Flavobacterium*, *Methylobacterium*, and *Agrobacterium*, whereas the probiotics significantly altered fewer genera in the overall microbial richness compared with the control group than the supernatant, affecting only *Paracoccus*, *Shimia*, *Roseivivax*, *Thalassobacter*, and SMB53.

Bacterial diversity was estimated with Shannon's index, a higher value of which indicates greater bacterial diversity [24, 34]. In this study, the values for Shannon's index suggested that the control group (Shannon index = 3.60) had greater community diversity than the H, N, or S groups (3.27, 3.31, and 3.47, respectively). Similar results were obtained by Luis-Villaseñor *et al*. [24], who reported that dietary supplementation with *Bacillus* mix significantly reduced the community diversity of the shrimp gut microbiota. These diversity data are similar to the results of Rungrassamee *et al*. [29], in which the highest Shannon index of the library was 3.46, much lower than that for humans (4.0) [1, 6] and mice (5.5) [12]. However, when the community diversity was estimated by the richness and evenness of the community, then based on the Chao/Ace ratio, the diversity analysis indicated that the evenness was lower in groups H and N than in group C. This contrasts with the report of Luis-Villaseñor (2013), who found that probiotics modulated the bacterial community in the intestine of *L. vannamei*, which was even more diverse than that of the control group [24], similar to the findings of Liu *et al*. [22]. The PCoA score plot and heat map showed that the intestinal samples from the same treatment did not cluster well within one group but clustered closely with samples from other groups, indicating fluctuations in the bacterial compositions of individuals in one group. This contrasts with the results of Rungrassamee *et al*. [30], who demonstrated that the bacterial composition of the shrimp gut microflora fluctuated less when reared under the same water conditions.

Taken together, these data show that the abundance of bacteria rather than the number of species was greatly influenced by dietary supplementation with probiotics and a probiotic supernatant, and indicate that the interactions between the different bacterial abundances in complex microbial communities may play an important role in maintaining or promoting the health of the host.

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