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Understanding the roles of surface proteins in regulation of *Lactobacillus pentosus* HC-2 to immune response and bacterial diversity in midgut of *Litopenaeus vannamei*

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ABSTRACT

The interactions of microbiota in the intestines play an important role in promoting or maintaining the health of hosts. The present study aim to investigate the effects of the surface proteins of *Lactobacillus pentosus* HC-2 on the immune response and the bacterial composition of *Litopenaeus vannamei*, thus, the immune-related genes, surface condition, HC-2 numbers and the bacteria diversity in midgut were explored after shrimp feeding the normal HC-2 and 5 M − lithium chloride (LiCl) treated HC-2 for four weeks. Obvious improvements in the intestinal surface were observed in R group than the control group and L group. qPCR analysis demonstrated that the selected immune-related genes of lysozyme, proPO, LGBP, PEN-3α, crustin, and lvLec were significantly up-regulated in group R than in group L. Meanwhile, in the challenge test, shrimp in R group received 72% relative percent survival, which was significantly higher than the L group (RPS = 9%). The bacteria composition analysis showed that the abundance of Proteobacteria were significantly higher in group R and L than in group C, and the Bacteroidetes were significantly higher in group C than in group R and L, whereas the numbers of Chloroflexi were significantly higher in group R than in group C and L. The bacterial community difference analysis revealed that the harmful bacteria such as genus of *Vibrio*, *Tenacibaculu* and *Thalassobius* were decreased and the beneficial bacterium as *Ruegeria* and *Lactobacillus* were increased in R group, whereas this phenomenon were not found in L group. Taken together, above results indicating that the surface proteins were indispensable for *L. pentosus* HC-2 adhesion and colonization in shrimp intestines to improve intestine condition, enhance immune response, competitively exclude the pathogens, and promote the beneficial bacteria growth to protect the shrimp from pathogens infection. The findings in this work will help to promote the understanding of the roles of probiotics in shrimp intestines displaying probiotic-function by regulating the intestinal bacteria.

1. Introduction

Litopenaeus vannamei is one of the most valuable crustacean species in capture fisheries and mariculture in the world [1,2], but their commercial production declines sharply with water environment deterioration, frequent disease outbreaks caused by viruses such as the white spot syndrome virus (WSSV), yellow head virus (YHV), and infectious hypodermal and hematopoietic necrosis virus (IHHNV) and by bacteria from the genus *Vibrio* [3,4]. In most situation, appropriate management of shrimp culture and maintaining balanced microbial communities among pathogenic, innocuous, and beneficial bacteria are help to prevent the bacterial disease outbreak [5]. The intestinal bacterial microbiota of farmed marine animals would be more strongly influenced by the food and the water environment [6]. Except the pathogens, actually, there are numbers of beneficial bacteria in the intestine, which contributing to nutrient absorption, immune responses, and epithelial development for the farming animals [7,8].

The probiotics were defined as live microbial feed supplements that improve health of animals [9]. In aquaculture, probiotics as one alternative to antibiotics have been commonly used for they confer several

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benefits, including immunological, nutritional, and environmental benefits [10,11]. Among the probiotics available, studies reported that some kinds of lactic acid bacteria mainly include *Lactobacillus. Delbrueckii, Lactobacillus. Plantarum, Lactobacillus. Helveticus, Lactobacillus. acidophilus, Lactobacillus pentosus* and *C. inhibens* have been widely administered due to they could significantly improve the immune status, evaluate the host digestion, modulate the bacterial community, and antagonize opportunistic pathogens [12–14]. In previous study, we isolated and identified a *L. pentosus* HC-2 from aquatic animals (*Acanthogobius hasta*), which have high antibacterial and adhesive activities to the intestinal mucus of *L. vannamei*, and the diet experiment shown that HC-2 could improve the immune responses, growth performance, and disease resistance of the *L. vannamei* compared with those of the controls [15]. To advance the utilization of *L. pentosus* HC-2 as a probiotic in the shrimp aquaculture, the mechanism of their probiotic action needed be explored. As known, adhesion is crucial and prerequisite step for Lactobacillus colonization to demonstrate their probiotic function in digestive tract [16], and the surface proteins have been considered to be involved in adhesion to the gastrointestinal epithelial cells and mucosa of mammals [17]. Actually, except the adhesive capacity, surface proteins also have the ability of maintain the shape of the bacteria, molecular sieve function, immunomodulation to the host, and providing extracellular enzyme binding sites [18–20]. However, our knowledge of the effects of surface proteins on the gut microbiota of invertebrates is unclear. 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.

Surface proteins usually bind to the outermost layer of the cell with non-covalent bonds, using denaturants lithium chloride (LiCl), guanidine hydrochloride (GuHCl), urea or metal chelating agents etc can depolymerize them to monomer [21–23]. Wherefore, in present study, we did a comparative analysis of intestinal surface structure, immune-related genes expression and bacterial composition of the intestinal microbiota in shrimp fed with normal *L. pentosus* HC-2 and LiCl-treated *L. pentosus* HC-2.

2. Materials and methods

2.1. Bacterial strains and growth conditions

Lactobacillus pentosus HC-2 (GenBank Accession No. KU995298) was isolated from the intestinal tract of fish (*Acanthogobius hasta*) and stored at - 80 °C in de Man, Rogosa, and Sharpe (MRS) broth (Qingdao Hope Biol-Technology Co., Ltd., Qingdao, China) containing 20% (v/v) glycerol [24]. For the experiments, the bacterial strains were grown unstirred in MRS medium at 37 °C under anaerobic conditions after inoculation with 1% (v/v) overnight culture and prepared as a fermentation seed culture.

2.2. Bacterial surface shaving

The bacteria were harvested on the transition between late exponential and stationary phase (OD600 ≈ 1.7) by centrifugation (3000 × *g*, 10 min, 4 °C). The cell pellet from 500 ml of culture was washed three times with 1 M phosphate-buffered saline (PBS) by centrifugation (3000 × *g*, 10 min, 4 °C) and subsequently resuspended in PBS containing 25% sucrose. Ten-fifteen mg of the moist pellet were suspended per ml of 5 M − lithium chloride (LiCl) and kept at 37 °C with shaking at 180 rpm for 9 h. After treatments, cells were collected by centrifugation (30,000 g, 10 min) and washed three times with autoclave sterilized seawater.

2.3. Diets preparation and feeding trials

In brief, cells were resuspended in sterilized seawater and sprayed on basal commercial feed at 5×10^8 colony-forming units (CFU) g/feed. 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.

The experimental *Litopenaeus vannamei* were obtained from Ruizi Seafood Development Co. Ltd. (Qingdao, China). Twelve aquaria (60 L) were used for triplicate experiments for each treatment, which involved three experimental diets. The shrimps weight 3.5 ± 0.06 g were selected for the experiments for the reason that the growth rate at this phase was relative faster, which help to obtain a significantly feeding effect. A total of 1200 healthy shrimps randomly divided into the 12 aquaria, each containing 10 shrimp. The shrimp were acclimated for 1 week with the control diet before the experiments commenced. To analyze the effects of strains HC-2 on the intestinal bacterial diversity and intestinal morphology of the shrimp, experiments were designed as follows: C group, shrimp fed a basal commercial diet alone as the control; R group, shrimp fed a basal commercial diet + strain HC-2 (5×10^8 CFU/g feed); L group, shrimp fed a basal commercial diet + LiCl treated strain HC-2 bacteria (5×10^8 CFU/g feed). The basal commercial 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. All shrimps were maintained in fresh seawater (salinity, 30‰) at 30 ± 2 °C with continuous aeration and a 50% water change every day. The daily feeding rate was 10% of the body weight. Animals were fed three times per day at 7:00, 12:00, and 18:00, 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.

2.4. Challenge tests

Vibrio parahaemolyticus E1 ATCC 17802 Strains was used as a pathogenic strain for the bacterial infection was donated by Doctor Wenbin Zhan (Laboratory of Pathology and Immunology of Aquatic Animals, Ocean University of China) saved in trypticase soy broth (TSB) containing 15% sterile glycerol solution and stored at −80 °C. *V.parahaemolyticus* E1was propagated aerobically in 2216E broth (Qingdao Hope Biol-Technology Co., Ltd) at 28 °C for 18 h. At the end of the feeding trials, 25 shrimp from each aquarium were transferred to a tank with 30 L of seawater containing 10^7 CFU/mL live *V. parahaemolyticus* E1 and were fed with basical diet. The mortality was recorded for three days post-challenge, and the relative percent survival (RPS) was calculated as previously described by Du et al. [25].

2.5. Scanning electron microscope

The appearance characteristics of intestines of *L. vannamei* after feeding for four weeks and challenged were examined using scanning electron microscope (SEM) according to the methods described by Valk et al. [26] with some modifications. Using the sterile forceps sampled the mid-intestines without washing and add 2.5% glutaraldehyde fixation (4 °C refrigerator overnight). Following, dehydrated in a series of 20, 40, 60, 70, 80, 90, 95, and 100% ethanol solutions (15 min each). Finally, the stubs were frozen at −80 °C overnight, CO_2 critical point freeze-dried for 4 h and sputter-coated with Gold palladium using Elko 1B.3 ion coater before viewing with the SEM (FESEM, MERLIN de Carl Zeiss, Oxford).

2.6. qPCR analysis of immune-related genes

To investigate the effects of experimental diets on the immunity of *L. vannamei*, the midgut from 8 shrimp per group (three per aquarium)

Table 1
Primers used in this study.

Target gene	Sequence (5′–3′)	Product size (bp)
β-Actin	F-GCCCATCTACGAGGGATA R-GGTGGTCGTGAAGGTGTAA	121
Lysozyme	F-TCTGCAAAATCCCATGTTCCG R-AGTTGGAACCGTGAGACCAG	198
ProPO	F-CGGTGACAAAGTTCCTCTTC R-GCAGGTCGCCGTAGTAAG	120
LGBP	F-TGGACGCTTATGTCACCTAC R-CTTCTACTTCATCTGTTGCT	177
PEN-3α	F-CTCGTGGTCTGCCTGGTCTTCTTG R-CAGGGCAACCGTTGTATGGA	151
Crustin	F-CGGAGTAGGTGTTGGTGGTGGTT R-CTCGCAGCAGTAGGCTTGAC	178
lvLec	F-GATCGAGGACTGCGAAACCT R-CCCCAGAAAGGTACACCCTG	162
pentF pREV	F-CAGTGGCGCGGTTGATATC R-TCGGGATTACCA AACATCAC	220

were sampled at two and four weeks post-feeding, and one day after challenge. RNA was extracted from tissues using an RNA fast extraction kit (Epicentre Biotechnologies, Madison, WI, USA) according to the protocol. Complementary DNA (cDNA) was synthesized with Reverse Transcriptase M-MLV kit (TransGen Biotech Co., Ltd., China).

The expression profiles of six immune-related genes lysozyme, proPO, LGBP, Penaeidins-3α (PEN-3α), crustin, and C-type lectin (lvLec) were investigated in the midgut using a Roche480 real-time PCR system (LightCycler 480, USA). *β*-actin was selected as endogenous control. Specific primers used in this study and the expected product size are listed in Table 1 qPCR was performed with the following two steps: denaturation at 94 °C for 30 s and then 40 cycles of 94 °C for 5 s and 60 °C for 30 s. The dissociation curve was harvested at the end of qPCR to confirm the specificity of PCR products. Dates were analyzed with Microsoft Excel, and the relative expression ratio (R) of mRNA was calculated according to $2^{-\Delta\Delta Ct}$ method.

2.7. Detection of the number of HC-2 in midgut by qPCR

The establishment of quantitative real time PCR (qPCR) standard curve for quantifying the number of HC-2 as described in Du et al. [27]. DNA was extracted from 4.994×10^9 cfu of *L. pentosus* HC-2 by DNeasy Blood & Tissue Kit (Qiagen, Germany), and diluted in 10-fold serial as templates to generate standard curve, equivalent to the cell number ranging from 4.994×10^9 to 4.994×10^1 cfu. PCR assay was performed by the specific Gene RecA primers: pentF and pREV [28] as list in Table 1. RNase free water was used as negative control, and each dilution with triplicate reaction. A total reaction volumes of 20 μl containing 2 μl (100 ng/ul) of DNA extraction, 10 μl of SYBR Green (Roche, Sweden), 1 μl of primers (10 μM), and 7 μl of RNase-free water. The cycling protocol was 94 °C for 5 min, 45 cycles of denaturation at 94 °C for 30 s, annealing at 60 °C for 10 s and extension at 72 °C for 10 s. DNA melting-curve analysis ensured that the desired amplicon was detected and specific. When Ct values (y) were obtained, the x values were calculated according to equation $Y = -3.1154x + 33.651$ ($R^2 = 0.9907$).

The midgut of eight shrimp from each group were taken in the one to four weeds post feeding experiment, and the total DNA were extracted to measure the HC-2 number in the tissues as above.

2.8. Extraction of DNA and Illumina-sequencing

The shrimps were starved for one day post 4-weeks feeding experiment, and the midguts of twenty shrimps from each tank were pooled into one sample, with triplicate samples per treatment. These samples were saved in liquid nitrogen for RNA and DNA extraction. The total genomic DNA was extracted using the ExtractMaster™Fecal DNA Extraction Kit (Epicentre Biotechnologies, Madison, WI, USA) following the manufacturer's instructions.

The diversity and composition of the bacteria in each sample were determined as the portocols described in Caporaso et al. [29]. Polymerase chain reaction (PCR) products were generated with the primer pair 338F (5′-ACTCCTACGGGAGGCAGCA-3′) and 806R (5′- GGACTACHVGGGTWTCTAAT -3′), which amplifies the V3 and V4 regions of the 16S rRNA gene. The products were sequenced with the Illumina Miseq platform. The short reads that had overlap were assembled using FLASH software [30] and the assembled reads of low quality were filtered through QIIME software [31]. In QIIME, poor-quality sequences were set as sequences with a length less than 150 bp, and contained ambiguous bases.

2.9. Taxonomic analysis and gene annotation

All the sequences were classified from phylum to species levels based on the RDP classifier [32] and taxonomic trees were constructed with MEGAN [33] using taxonomic assignments. The reads acquired were clustered with the QIIME software based on a similarity of at least 97% 16S rRNA sequence self-similarities to obtain more information about the bacterial diversity instead by matching them to an external database, and were classified into operational taxonomic units (OTUs) [34]. Chao biodiversity index, Shannon index, ACE index, and Simpson index values were calculated for each shrimp library using MOTHUR [35]. Beta diversity analysis was used to compare the microbial community compositions in the different samples with QIIME [31], including weighted and unweighted UniFrac. The taxonomic information was annotated by a naive Bayesian model using RDP classifier (Version 2.2) based on SILVA [36] Database (Release115, http://www.arb-silva.de). A principal components analysis (PCA) and a principal coordinate's analysis (PCoA) were applied to genotype data based on the unweighted UniFrac distance. The cluster analysis and heat map production of the first 50 genera of abundance were conducted using R software. The Metastats software was used to screen the biomarker features on the difference of sequence quantity (absolute abundance) in the taxa levels of the phylum and the genera.

2.10. Statistical analysis

Data representing the abundances of the OTUs were analyzed with the SPSS software (ver. 17.0, IBM Corp, Armonk, NY, USA). One-way analysis of variance was used to analyze the differences among the different treatment groups. $p < 0.05$ was considered significant. The data is all presented as mean ± standard error (SE).

3. Results

3.1. The intestinal surface structure of L. vannamei and relative percent survival (RPS)

The appearance characteristics of intestines of *L. vannamei* after feeding with normal HC-2 and LiCl-treated HC-2, and the changes after the shrimps were challenged by *V.parahaemolyticus* E1 were observed by Scanning Electron microscope (Fig. 1). The electron-graph showed that the chorionic layer of intestines were enrich covered by mucus after the shrimp fed with normal HC-2 (Fig. 1B), on the contrary, copious amounts of feces were cover on the surface intestines after the shrimp fed with basical diet or suppled with LiCl-treated HC-2 (Fig. 1A and C). After the shrimp were challenged by the *V.parahaemolyticus* E1, the mucus covered on the group R shrimp's intestines surface decreased (Fig. 1E), but the chorionic layer were integrity, for group L and the control group, the intestinal chorionic layer of shrimp were destroyed where showed became more uneven (Fig. 1D and F). The cumulative survival rates of shrimp after challenge with live *V.parahaemolyticus* E1 and the relative percent survival (RPS) in different experimental groups

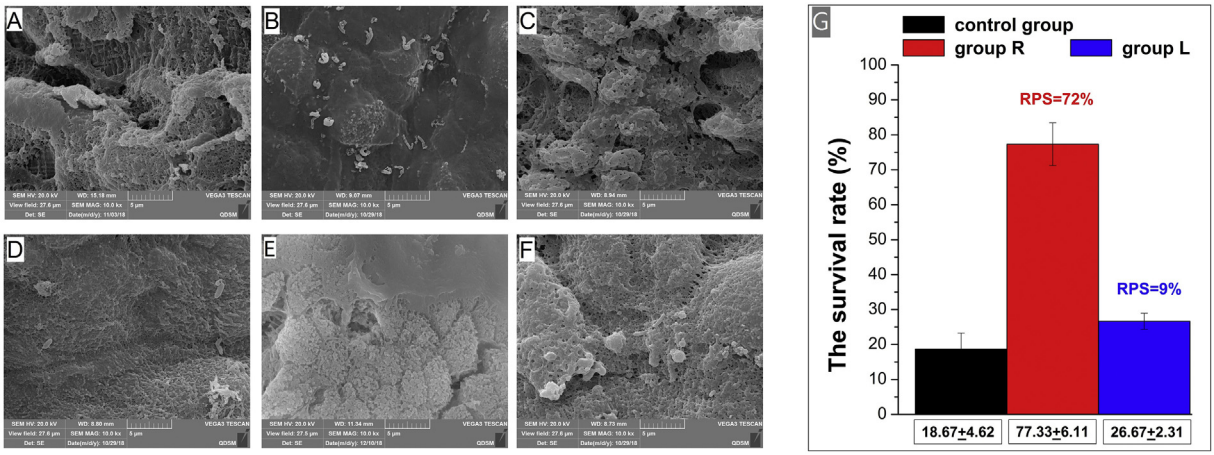


Fig. 1. The intestinal surface structure and relative percent survival of *L. vannamei*. A: Gut surface of shrimps were fed a basic diet; B: Gut surface of shrimps were fed a basic diet supplied with normal *L. pentosus* HC-2; C: Gut surface of shrimps were fed a basic diet supplied with LiCl-treated *L. pentosus* HC-2; D, E and F were showed the intestinal surface structure of shrimps in A, B and C respectively which were challenged by *Vibrio parahaemolyticus* E1. G: The relative survival rate of shrimp after challenged with *V. parahaemolyticus* E1 at the four weeks post feeding.

are shown in Fig. 1G. In the control group, the challenged shrimp appeared obvious red tail and hepatopancreas atrophia symptom and the cumulative survival rate was 18.67% post challenged for three days. In the shrimp group feeding with normal HC-2 had relative survival rate of 72%, however, the shrimp fed with LiCl-treated HC-2 only got 9% of RPS which were significantly lower than the group R.

3.2. Expression of immune-related genes and numbers of HC-2 in the midgut

After feeding with normal or LiCl-treated HC-2 both induced the immune-related genes expression compare with the control (Fig. 2). All the six detected genes except crustin were significantly up-regulated from two weeks after shrimp fed with normal HC-2 compared with the control, the expression level even significantly higher than the shrimp fed with LiCl-treated HC-2. Meanwhile, the transcription level of proPO, LGBP, and lvLec were significantly increased at four weeks post in group L. After the shrimp were challenged by *V.parahaemolyticus* E1, the detected genes were significantly increasing in group R and L, especially the proPO and lvLec genes expression in group R (Fig. 2C and F). However, the ability of resistance to *V.parahaemolyticus* E1 in the shrimp fed with LiCl-treated HC-2 were much lower than the shrimp fed with normal HC-2 that the six immune genes expressed significantly lower in group L than in group R.

The numbers of HC-2 in the midgut has also been measured by qPCR during the feeding experiment. In the control group, the HC-2 were most can't be detected (Fig. 3D). After the shrimp feeding with normal HC-2, the numbers of HC-2 were increasing from one week, and from two weeds the population of HC-2 were dramaticly increasing to 10^6 cfu at four weeks. However, the HC-2 amount in midgut of shrimp fed with LiCl-treated HC-2 were significantly lower than in group R shrimp.

3.3. Sequences obtained

Aim to analysis the bacterial microbiota, 16S rRNA gene amplicons from the intestinal samples from shrimps of groups C, R, and L were sequenced with Illumina. A total of 672,840 sequences of the V3—V4 region of the 16S rRNA gene were obtained with Illumina sequencing and with an average read length of 383 ± 68 bases (Table 2). All sequences were sorted with a barcode for each group: C (45,315 sequences), R (50,082 sequences) and L (51,127 sequences).

3.4. Richness and diversity

The obtained sequences were divided into OTUs merge at a dissimilarity level of 0.03, and the representative sequences of each OTU were used for classification status identification and phylogenetic analysis. The total number of OTUs was highest in group L (8136 OTUs), whereas groups C, and R contained 7423 and 7683 OTUs, respectively (Table 2). A rarefaction analysis was performed to determine whether the current sequencing depth of each sample is adequate to reflect the microbial diversity in the community sample. These curves showed that all the samples reached saturation for the three treatment groups even in most of the libraries that contained > 25,000 reads, suggesting that no more bacterial species are expected in shrimp intestines (Fig. 4A). The community richness (Table 2) 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 C than in groups R and L, indicating that the bacterial community richness was higher in group C than in group R and L group (C > R > L). Bacterial community diversity was assessed with Simpson's and Shannon's indices. The value for Simpson's index was highest in group C, following in group R, and lowest in L group (C > R > L). However, Shannon's index was higher in group C than in groups L, and R group was the lowest (C > L > R).

In order to study the differences in OTUs between different groups, Venn diagram of the OTUs at the 0.03 dissimilarity level was constructed to identify the common or specific information of OTUs presented in intestines (Fig. 4B). As the results showed that in total 4, 654 species of bacteria were found in all the samples in the three groups, and there were distinct differences among the microbial structures of the three groups. The experimental group R had the greatest number of unique species (876 in total) than the group L (830 in total) and the control group (623 in total).

3.5. Bacterial composition and community structure

Based on the RDP classifier, the highest number of bacteria phyla was detected in samples from group L, whereas the samples from group C and group R had the equal number of bacterial phyla (16, 15, and 15 phyla for groups L, C, and R, respectively). The top 10 phyla and their relative abundance are shown in Fig. 5. Proteobacteria were the predominant microflora in the three groups at the phylum level, it account for more than 72.226% of all the bacterial communities in group C, while, in group R and L, the proportion were as high as 83.244% and 81.759% which were significantly higher than in group C. Another nine

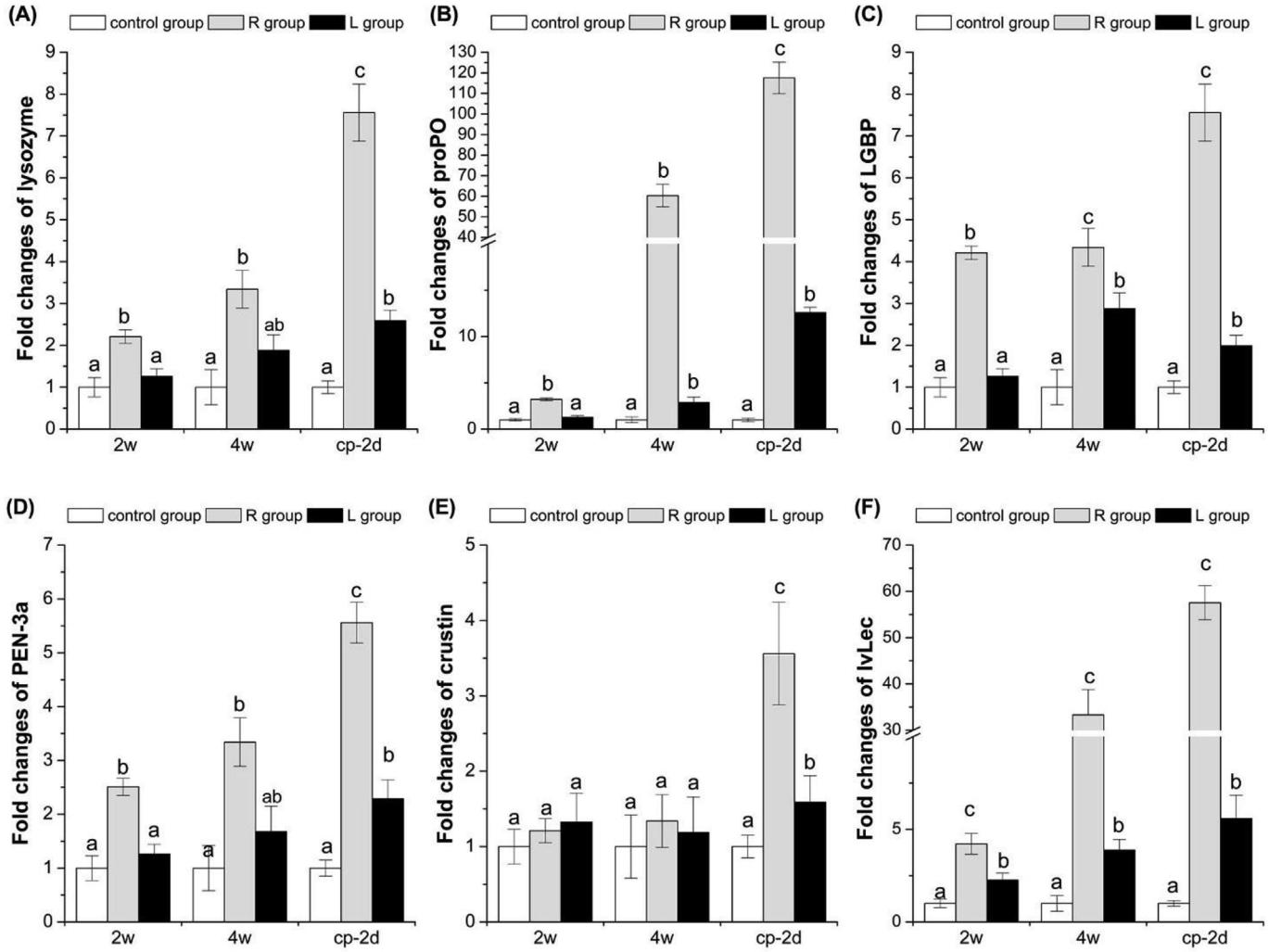


Fig. 2. Relative expression of the immune-related genes (lysozyme, proPO, LGBP, PEN-3α, crustin, and lvLec) in the midguts of L. vannamei at two and four weeks, and challenged two days (cp-2) post-feeding measured by SYBR green qPCR. The letters above the bars that are not the same are significantly differentat$P \leq 0.05$ based on ANOVA.

microflora followed by Bacteroidetes, Tenericutes, Actinobacteria, Firmicutes, Verrucomicrobia, Fusobacteria, Chloroflexi, Acidobacteria and Planctomycetes. Among them, the abundance of Bacteroidetes was highest in group C (25.387%), but did not differ significantly between group R (7.821 ± 4.886) % and group L (11.486 ± 0.953) %. Additionally, the abundance of Chloroflexi in group R (0.762%) was significantly higher than in group C and in group L. The other seven phyla were not differ among the three groups.

The top 20 genera and their relative abundance are listed in Table 3. *Photobacterium*, *Vibrio*, *Ruegeria*, uncultured *Rhodobacteraceae* and *Tenacibaculum* were the predominant genus in group C, R and L. The relative abundance of *Photobacterium* was higher in group R and L than in group C, but their differences were not significant. The abundance of *Vibrio* in the control group displayed significantly higher than in group R, but not showed significantly higher than group L. The abundance of *Ruegeria* in group R showed significantly higher than in group C and in group L. The control group own the highest relative abundance of *Tenacibaculum*, following group L, which were significantly higher than group R. *Thalassobius* taken higher population in control group than in group R and L. Interestingly, the *Lactobacillus* were detected in the three groups, and the group R had the highest relative abundance than the group L and C, but the relative abundance in control group was truly much lower. In other genus, they did not differ significantly in their relative abundance among the three groups.

3.6. Relationships among the bacterial communities

The first fifty genera of abundance were performed clustering analysis, the heat maps were constructed at genus level based on the bacterial communities of all 9 samples. In the cooperation of group C and R or L group, both were clearly grouped into two categories (Fig. 6). However, when compare the group R with group L, the analysis showed that the samples segregated into three groups, one group was mainly composed of subgroups L3, L1 and R2, and another group was composed mainly of samples in groups R3 and R1, and group C3, C1, C2 and L2 were grouped into a category. A PCoA was performed to determine the relationships among the groups (Fig. 7). Samples of group C were clustered on the left-hand side of the graph, along the second principle component axis (PC1), whereas samples of group R clustered on the right hand side of the graph. However, samples of group L 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, R and L differed and that the bacterial community in the intestines was influenced by the addition of probiotics or LiCl-treated probiotics. The significantly different phyla and genera showed in Fig. 8 & Fig. 9 confirm this.

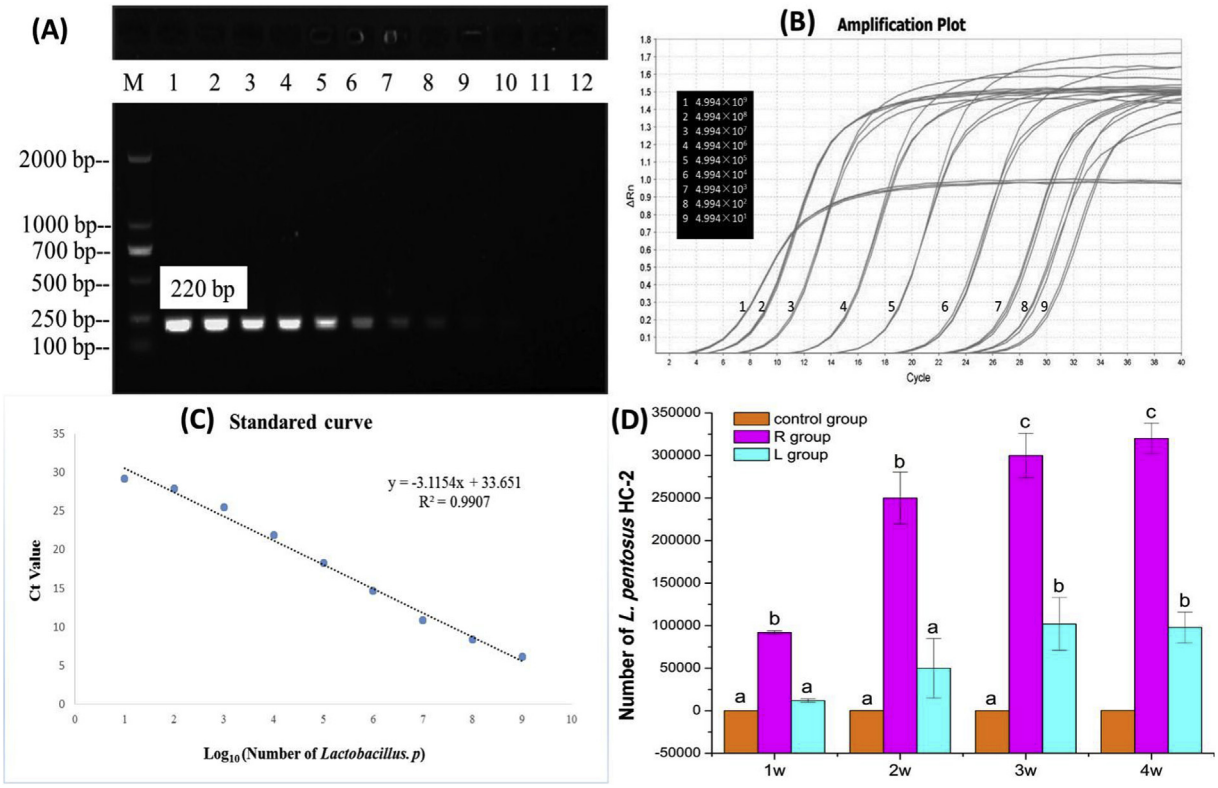


Fig. 3. Measurement of the numbers of *L. pentosus* HC-2 in midgut of Shrimps. (A): Agarose gel electrophoresis of the specific RecA primer PCR production. (B): Sensitivities of qPCR for detection of HC-2 after 45 cycles, Curves 4.994×10^9 to 4.994×10^1 cfu HC-2. (C): The standard curve generated from the threshold cycle values (y) against $\log_{10}$ (Number of HC-2 (x)). (D): The numbers of HC-2 in shrimp midguts post fed a basal diet (C) or a basal diet supplemented with normal HC-2 (R), LiCl-treated HC-2 (L) at one to four weeks. Values were means ± SE, and different letters denoted significant difference among different immersion groups at the same sampling time ($P < 0.05$).

Table 2
The assignment and analysis of sequences.

	Different treatments		
	C	R	L
Sampling depth			
Total sequences	45,315	50,082	51,127
OTUs	7423	7683	8136
Phylum	15	15	16
Class	26	30	29
Order	45	56	50
Family	72	92	80
Genus	138	162	153
Species	164	186	176
Diversity index and estimated OUT richness			
Simpson	0.970 ± 0.017	0.963 ± 0.012	0.967 ± 0.006
Chao1/ACE	0.987 ± 0.023	0.980 ± 0.020	0.977 ± 0.015
Shannon	7.563 ± 0.464	7.303 ± 0.201	7.440 ± 0.312

Shrimps were fed with a basal diet (C) or a basal diet supplemented with normal *L. pentosus* HC-2 (R), a basal diet supplemented with LiCl-treated *L. pentosus* HC-2 (L).

3.7. Differential analysis of bacterial communities

According to the composition and sequence distribution of each sample at each taxonomic level, Metastats was performance to compare the abundance differences of each classification unit among the three groups and statistical tests evaluate the significance of the difference. The statistic results showed in Fig. 8 (phylum) and Fig. 9 (shown top 20 different genus). In phylum, the absolute abundance of Acidobacteria, Chloroflexi, Firmicutes, Proteobacteria and SBR1093 in group R were markedly higher than in the control group, and the Bacteroidetes in group R were lower than in group C (Fig. 8A). The abundance of Bacteroidetes, Firmicutes, Fusobacteria, Proteobacteria, Verrucomicrobia and WS6 had difference between group L and C, and the abundance of Bacteroidetes and Fusobacteria were significantly higher in group C than in group L (Fig. 8B). Surprisingly, compared with group R, the group L had lower in the abundance of Acidobacteria, Chloroflexi, Firmicutes and WS6 (Fig. 8C). The results of genus difference analysis among the three groups showed that the abundance of *Algicola*, *Aquamicrobium*, *BD1_7_clade*, *Halomonas*, *Labrenzia*, *Lactobacillus*, *Loktanella*, *Mesorhizobium*, *Paracoccus*, *Roseovarius_sp._HMF2302* and *Regeria* were significantly higher in group R than in group C, and *Alibacter*, *Donghicola*, *Lacinutrix*, *Olleya*, *Polaribacter_2*, *Polaribacter_4*, *Psychroserpens*, *Tenacibaculum* and *Vibrio* were significantly lower in group R than in the control group (Fig. 9A). The abundance of *Albidovulum*, *Haloferula*, *Lactobacillus*, *Planktomarina*, *Plantotalea*, *Pseudoruegeria*, *Sagittula* and *Subsaximicrobium* were significantly higher in group L than in group C, whereas the *Ascidiaceihabitans*, *Candidatus_Microthrix*, *Cetobacterium*, *Flavobacterium_sp._MS_3*, *Jannaschia_sp._KYW137*, *Lacinutrix*, *Leisingera*, *Olleya*, *Polaribacter_2*, *Polaribacter_4*, *Sulfitobacter* and *Tenacibaculum* were significantly lower in group L than in control group (Fig. 9B). Especially, the abundance of *Lactobacillus*, *Leisingera*, *OM60 (NOR5)_clade*, *Poseidonocella* and *Ruegeria* were significantly higher in group R than in group L, and *Tamlana* and *Tenacibaculum* were significantly lower in group R than in group L (Fig. 9B).

4. Discussion

The bacterial microbiota in the digestive tracts have many roles in animal's health, most of which are beneficial or neutral for the host, including in nutrient absorption, immune responses, and modification of the mucosal and gut morphology [37,38]. The colonic microflora is a

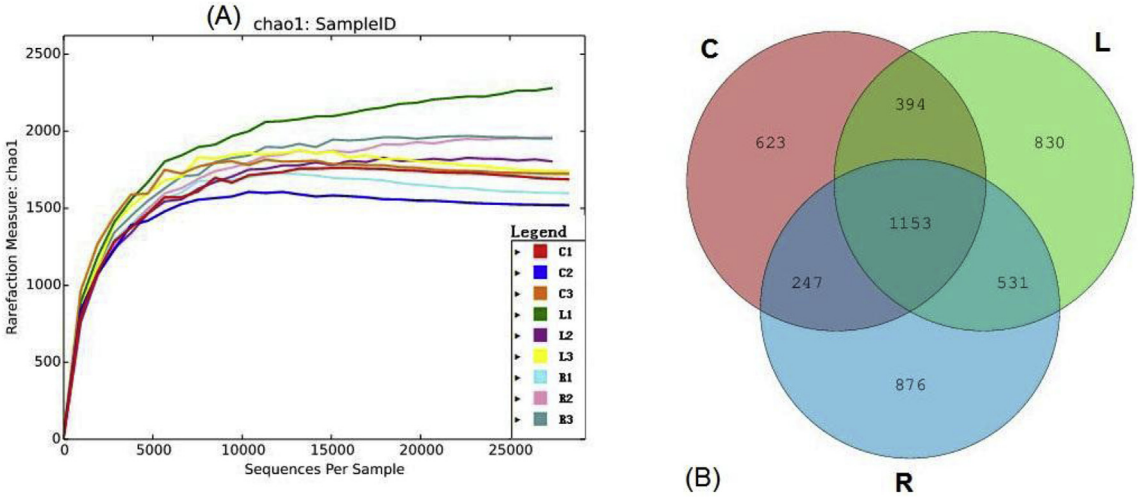
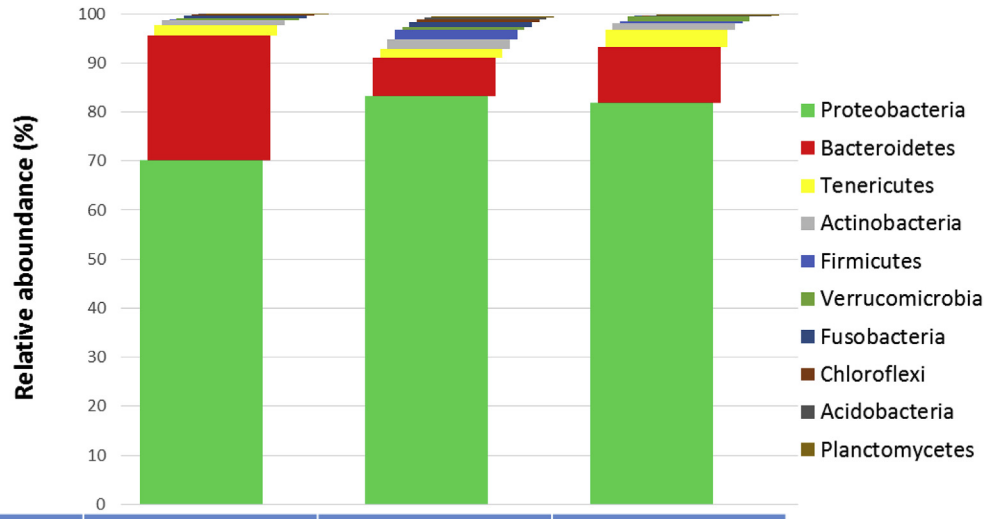


Figure 4. (A): Rarefaction analysis of shrimp intestines. The number of sequences represents the number of Illumina sequencing reads. (B): Venn diagram showing the unique and shared operational taxonomic units (OTUs) among the three treatment groups. Shrimps were fed with a basal diet (C) or a basal diet supplemented with normal *L. pentosus* HC-2 (R), a basal diet supplemented with LiCl-treated *L. pentosus* HC-2 (L).

complex bacterial ecosystem of different animals, which are easily affected by the genetic background, diet, and environment [39]. Recent years, increasing attention has been paid to the utilization of Lactic acid bacteria as probiotic to regulate the intestinal microbial communities to defend the shrimp gastro-intestinal disease [40,41]. Our previous work had investigated the effects of *L. pentosus* HC-2 on the growth, immune response and intestinal microbiota of *L. vannamei* by feeding with the normal L. pentosus HC-2 [15,42], and the purpose of this work was to



Phylum	C (%)	R (%)	L (%)
Proteobacteria	70.226^{a} ± 0.847	83.244^{b} ± 1.124	81.759^{b} ± 2.461
Bacteroidetes	25.387^{b} ± 0.726	7.821^{a} ± 4.886	11.486^{a} ± 0.953
Tenericutes	2.089 ± 1.332	1.762 ± 2.287	3.488 ± 1.555
Actinobacteria	0.958 ± 0.327	1.896 ± 1.029	1.279 ± 0.173
Firmicutes	0.023 ± 0.021	1.927 ± 0.185	0.521 ± 0.204
Verrucomicrobia	0.317 ± 0.039	0.593 ± 0.350	0.802 ± 0.139
Fusobacteria	0.630 ± 0.186	0.922 ± 0.756	0.107 ± 0.137
Chloroflexi	0.120^{a} ± 0.045	0.762^{b} ± 0.246	0.068^{a} ± 0.039
Acidobacteria	0.006 ± 0.010	0.325 ± 0.301	0.067 ± 0.012
Planctomycetes	0.084 ± 0.036	0.140 ± 0.141	0.157 ± 0.014

Fig. 5. Relative abundance of the top 10 phyla in the three experimental groups. Shrimps were fed a basic commercial diet (C) or a basic commercial supplemented with *L. pentosus* HC-2 (R), a basic commercial supplemented with LiCl-treated *L. pentosus* HC-2 (L).

Table 3
The 20 major genus in the shrimp intestine.

Genus	Different Treatments		
	C (%)	R (%)	L (%)
Photobacterium	11.148 ± 1.697	14.891 ± 4.323	19.584 ± 8.512
Vibrio	18.450^{b} ± 2.907	10.054^{a} ± 1.384	15.045^{ab} ± 4.077
Ruegeria	5.940^{a} ± 0.428	16.125^{b} ± 2.451	8.767^{a} ± 2.674
uncultured Rhodobacteraceae	7.053 ± 0.675	9.612 ± 1.888	7.148 ± 0.184
Tenacibaculum	14.628^{b} ± 2.674	0.751^{a} ± 0.586	3.440^{a} ± 0.624
Roseovarius	2.547 ± 0.117	3.328 ± 0.498	2.877 ± 0.683
unidentified Rhodobacteraceae	2.769 ± 0.048	2.980 ± 0.521	2.675 ± 0.671
Candidatus_Bacilloplasma	2.016 ± 1.300	1.717 ± 2.310	3.248 ± 1.514
Flavobacterium_sp._MS-3	3.209 ± 0.938	2.378 ± 1.711	1.079 ± 0.279
Pseudoalteromonas	1.437 ± 1.030	1.491 ± 0.672	3.535 ± 1.970
Catenococcus	1.418 ± 0.266	0.927 ± 0.160	1.750 ± 0.601
Halocynthiibacter	1.496 ± 0.315	0.787 ± 0.215	1.669 ± 1.264
Haliea	0.412 ± 0.091	1.945 ± 1.078	0.792 ± 0.545
Pseudoruegeria	0.530 ± 0.064	1.350 ± 0.610	1.125 ± 0.158
Kriegella	0.507 ± 0.218	1.447 ± 0.963	0.744 ± 0.318
Thalassobius	1.144^{b} ± 0.239	0.772^{ab} ± 0.171	0.616^{a} ± 0.182
Maribacter	0.878 ± 0.317	0.565 ± 0.413	1.006 ± 0.186
Pseudoroseovarius	0.905 ± 0.301	0.840 ± 0.149	0.693 ± 0.377
Lactobacillus	0.009^{a} ± 0.015	1.854^{c} ± 0.128	0.415^{b} ± 0.105
Roseobacter_clade_CHAB-I-5_lineage	0.881 ± 0.155	0.715 ± 0.038	0.631 ± 0.072

Shrimps were fed with a basal diet (C) or a basal diet supplemented with normal *L. pentosus* HC-2 (R), a basal diet supplemented with LiCl-treated *L. pentosus* HC-2 (L).

further research the roles of the surface proteins in the process of probiotic function of *L. pentosus* HC-2.

The electron microscopy observation found that feeding with normal HC-2 improved the shrimp intestine surface condition, which was beneficial to protect the chorionic layer from the pathogen damage, but not found in the shrimp fed with LiCl-treated HC-2. As known, the antimicrobial peptides (AMPs), the clotting cascade and the proPO system were most important component parts in the humoral immune responses of invertebrates, which play prominent roles in host defence to pathogens [43–45]. LGBP was an important pattern recognition receptors (PRRs), which up-regulated when shrimp are challenged with *Vibrio alginolyticus* [46]. Many reports suggested that C-type lectin would up-regulated in shrimp when challenged with pathogenic bacteria [47,48], which contributing to protect the host from diseases. In present work, after feeding and challenge treatments, the selected immune-related genes of lysozyme, proPO, LGBP, PEN-3α, crustin, and lvLec were significantly increased higher in R group than that in control and L group. Meanwhile, the R group got significantly higher relative percent survival than the L group in the challenge experiment. These results demonstrated that surface proteins of HC-2 play an important role in the regulation process of shrimp immune response to against pathogens. In addition, qPCR showed that the numbers of HC-2 increased significantly higher in R group than in L group, which meaning that surface proteins of HC-2 were necessary for HC-2 adhesion and colonization in shrimp midgut.

In order to explore the influence of surface proteins of HC-2 on the shrimp intestinal microorganism, the bacterial composition was characterized using Illumina sequencing. Although the data showed that each group contained 7423–8136 OTUs which were much more than our previous detected, the phyla quantity (15–16 phyla) were significantly littler than before determine (26–29 phyla) [24], and many more than were determined in wild caught *Penaeus monodon* [49,50], in which had the highest number of OTUs (806) detected with 454 pyrosequencing. These diversities may result from the different sequencing techniques applied, and also from the different species of shrimp examined or the culture conditions used. While, several OTUs were found to be shared in the *L. vannamei* intestines of three groups, among which Rhodobacteraceae (family), belonging to the Proteobacteria (phylum), were dominant in all the samples, but the quantity of group R and L were significantly higher than group C, and no difference between group R and L, suggesting that supplement of HC-2 or LiCl-treated HC-2 had led to the change of major colony structure of shrimp intestinal bacteria. This results were consistent with previous reports that Proteobacteria were the most prevalent members in the shrimps, as in sea cucumbers and fish [6,49,51]. Illumina sequencing data suggested that the ten dominant bacteria phyla were Proteobacteria, Bacteroidetes, Tenericutes, Actinobacteria, Firmicutes, Verrucomicrobia, Fusobacteria, Chloroflexi, Acidobacteria and Planctomycetes. Consistently, these bacterial phyla were also determined in previous work by Sha et al. [42] and Hou et al. [52], and in intestine of *Penaeus monodon*, Penaeus notialis [53,54], which indicated that the similar bacterial community compositions were in shrimp intestine. Though the dietary supplementation with probiotics or LiCl-treated probiotics didn't significantly influence the types of phyla compared with the control, altered the population such as increased the numbers of the Proteobacteria in group R and L compared with the control, and decreased the amount of the Bacteroidetes in group R and L, and significantly increased Chloroflexi bacteria in group R. The abundance of others phylum as Actinobacteria, Firmicutes, Verrucomicrobia, Fusobacteria, Acidobacteria and Planctomycetes were all higher in group R and L than in the control group, but the difference was not significant. It's worth noting that Chloroflexi was a group of bacteria that produce energy through photosynthesis, which may have been associated with the better growth performance.

Interestingly, in this study, *Lactobacillus* were detected in group R and L by Illumina sequencing, but in the control group the abundance was very low, almost undetectable, especially the abundance were significantly higher in group R than in group L, which indicated that the *Lactobacillus* detected in the shrimp midguts of group R and L came from the diet and many more *Lactobacillus* adhered and colonized in the intestine of *L. vannamei* after fed with HC-2, and the surface proteins did affected the adhesion and colonization of HC-2. Note worthily, no *Lactobacillus* were detected in precious work [42], it probably because the amount of feeding bacteria is too low (10^{7} CFU/g) than in this work (5×10^{8} CFU/g), which hinder the *Lactobacillus* to be the dominant microflora in the shrimp intestines. Among the top twenty genus, *Photobacterium* and *Vibrio* were belonging to the Vibrionaceae (family), and were regarded as the most common pathogen for bacteria disease during shrimp aquaculture [55]. The comparative analysis results of relative abundance in genus levels showed that *Photobacterium*.spp

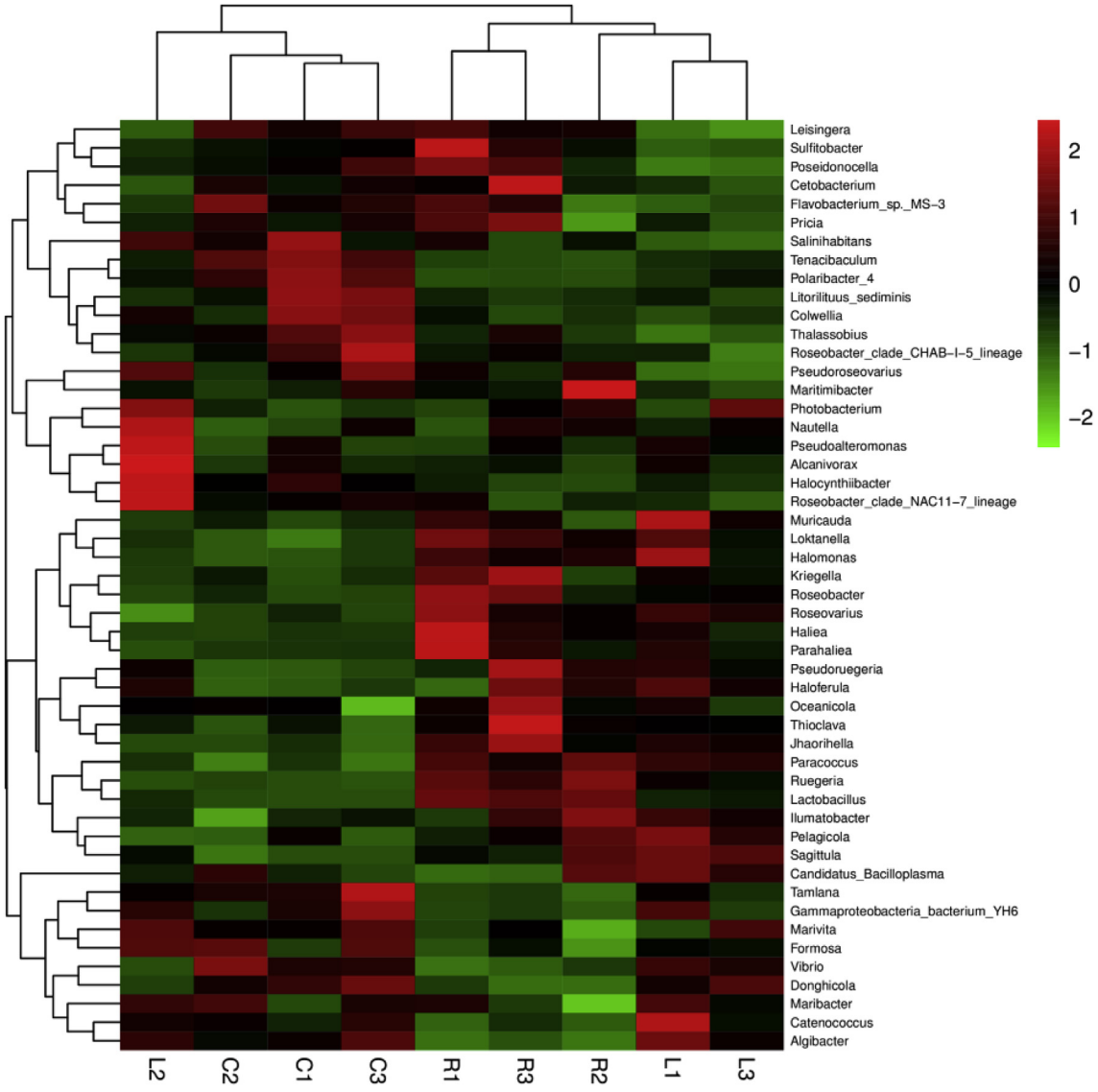


Fig. 6. Heat map of the bacterial diversity in the 9 samples of shrimp. Shrimps were fed a basal diet (C [C1, C2, C3]) or a basal diet supplemented with *L. pentosus* HC-2 (R [R1, R2, R3]), LiCl-treated *L. pentosus* HC-2 (L [L1, L2, L3]).

were much higher in group L than in group R and group C, but they didn't differ significantly. While, the *Vibrio* number was significantly decreased after the shrimp fed with HC-2, whereas the shrimp fed with LiCl-treated HC-2 didn't significantly decrease the *Vibrio* number compared with the control group. On the one hand, feeding with normal HC-2 improved the shrimp intestine surface structure protecting the chorionic layer avoiding the pathogen damage and enhanced the shrimp immune response to protect against pathogens as Vibrio. On the other hand, our previous work had been found that the population density of *V. parahaemolyticus* E1 decreased significantly after coculture with *L. pentosus* HC-2, suggesting that *L. pentosus* HC-2 competitively excluded *V. parahaemolyticus* E1 in the intestine of *L. vannamei* when the shrimp diet contained both these strains, and also found that LuxSis of *L.pentosus* HC-2 involved in the competitive exclusion of *V.parahaemolyticus* E1 by *L.pentosus* HC-2 [24]. Thus, the present work results suggest that maybe because shaving off surface proteins decreased the cell adhesion and colonization in the shrimp intestines and resulted in *L. pentosus* HC-2 couldn't competitive exclusion *Vibrio spp*. However, the mechanism that which proteins and how to regulate the competition-interaction need more study. In addition, it was reported that Ruegeria was a globally distributed Gram-negative marine bacterium could produce antibacterial compound tropodithietic acid (TDA) and biofilm formation against many marine pathogens and has strong N-acylhomoserine lactones (AHLs) degradative activity, and thus could be considered as a potential probiotic bacterium [56–58]. In present work, the abundance of *Ruegeria* spp. were significantly higher in group R than in group L and in control group, which indicating that shrimp fed with HC-2 could increase the probiotic bacterium. *Tenacibaculum* spp. was the domain bacteria belong to Bacteroidetes in the control group, which were significantly higher than in group R and L. However, *Tenacibaculum* spp. were commonly pathogitical bacteria which were serious threat to shrimp farming [59]. Thus, the lower abundance of *Tenacibaculum* spp. in group R suggested that *L. pentosus* HC-2 could competitive exclusion of *Tenacibaculum* spp. Species of Pseudoalteromonas are usually found in association with marine animal and display anti-bacterial, bacteriolytic, agarolytic and algicidal activities [60]. The abundance of Pseudoalteromonas spp. were higher in group L than in group R and in group C, but didn't differ significantly.

According to the absolutely abundance level of genus analysis, the impact of dietary supplementation with HC-2 or LiCl-treated HC-2 is greater. *Loktanella* spp. was also a larger population were significantly higher in group R compared with group C, which was reported can

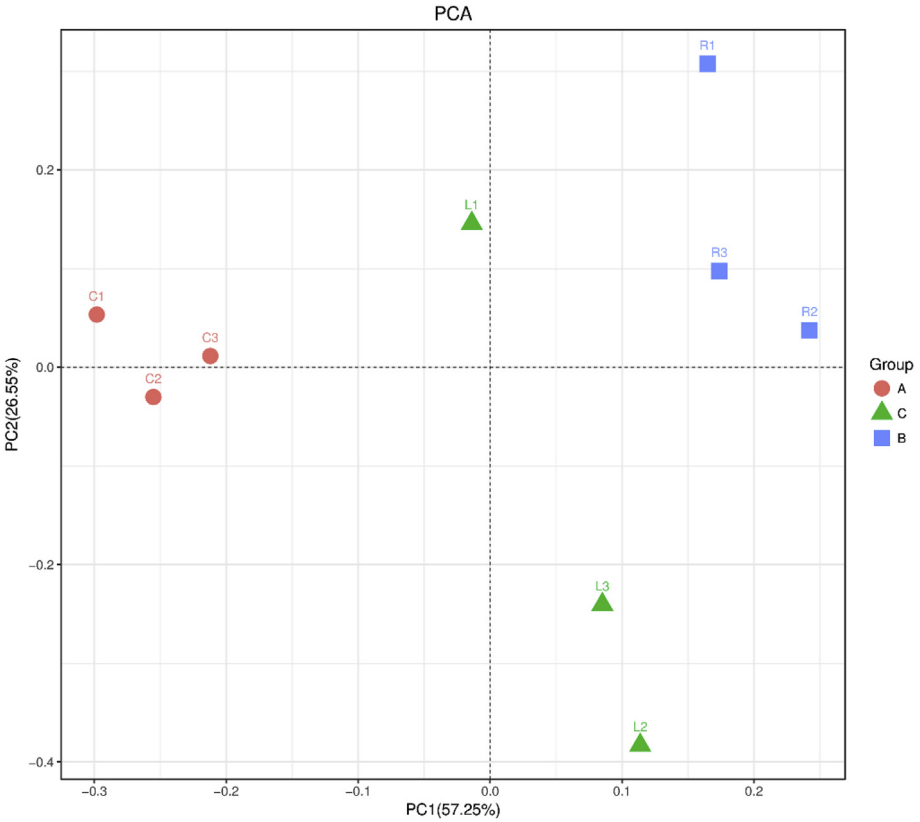


Fig. 7. Principal coordinates analysis scores based on the Unifrac distance. PC1: the first principle component; PC2: the second principle component. Shrimps were fed a basal diet (C) or a basal diet supplemented with *L. pentosus* HC-2 (R), LiCl-treated *L. pentosus* HC-2 (L).

survive under temperature extremes and inhibits the growth of the shrimp pathogen *Vibrio parahaemolyticus* when co-cultivated on an agar surface, as determined by a zone of inhibition assay [61]. There have been reported that *Sulfonamide antibiotics* (SAs) are the most commonly used antibiotics in human and veterinary medicine, and the residues of SAs in water, sediments, and soils have potential hazardous impacts on human health and natural ecosystems, however, Yang et al. [62] recently found *Aquamicr*obium were major bacterial communities involved in Sulfonamide antibiotics (SAs) degradation, so have potential application in improving water quality. *Polaribacter spp*. and *Sulfitobacter sp*. were among the most abundant and frequently occurring during phytoplankton bloom in an Antarctic polynya as reported by Choi et al. [63]. But in present study found that *Polaribacter spp*. were significantly higher in group R than in group C, and *Sulfitobacter spp*. were significantly higher in group L than in group C. *Flavobacterium spp*. the causative agent of bacterial coldwater disease (BCWD) is a significant threat to global aquaculture [64]. This work found that *Flavobacterium spp*. were abundant bacteria in the group C that significantly higher than in group L, but not differ significantly within group R, which demonstrated that shrimp fed with LiCl-treated *L. pentosus* HC-2 could decrease some specific pathogenic bacteria. In particular, among the top twenty significantly different genus, about thirteen genus were significantly higher in group R than in group L.

Bacterial diversity was estimated with Shannon's index, a higher value of which indicates greater bacterial diversity [65]. In this study, the values for Shannon's index suggested that the control group (Shannon index = 7.563 ± 0.464) had greater community diversity than the R or L groups (7.303 ± 0.201 and 7.440 ± 0.312, respectively), and group R had the lowest community diversity. Similar results were obtained by Sha et al. [42] and Luis-Villaser et al. [66] who reported that dietary supplementation with Bacillus mix significantly reduced the community diversity of microbiota in the shrimp intestines. Furthermore, when using the Chao/Ace ratio estimate the richness and evenness of the community diversity, the diversity analysis indicated that the evenness was also lower in groups R and L than in group C. This contrasts with the report of Sha et al. [42], but consist with the study of Luis-Villaser [66], who found that probiotics regulated the bacterial community in the *L. vannamei* intestine, which was even more diverse than that of the control group. The heat map analysis were consist with the PCoA score plot statistics which showed that the intestinal samples from the same treatment did cluster well within one group except the treatment of group R2 and L1, indicating fluctuations in the bacterial compositions of individuals in group R and L. If, except to analysis methods difference, the fluctuations may be caused by the different supplemental diet.

5. Conclusion

Taken together, these results showed that feeding with normal HC-2 could improve the chorionic layer of shrimp intestines avoiding pathogen damage and the immune response to against diseases, but feeding with LiCl-treated couldn't protect the chorionic layer from the pathogen damage. Also, the numbers of adhesion and colonization HC-2 in the midgut were much higher in the shrimp fed with normal HC-2 than in the shrimp fed with LiCl-treated HC-2. The abundance of bacteria rather than the number of species was greatly influenced by dietary supplementation with normal HC-2 and LiCl-treated HC-2. The dietary supplement with normal HC-2 had increased the abundance of beneficial bacterium which has ability of enhancing the immune response, improving the nutrition growth and producing adhesion factors to shrimp and decreased the harmful bacteria, however, when the shrimp fed with LiCl-treated HC-2 didn't promoting beneficial bacteria growth or decreasing pathogens populations than fed with normal HC-2. Therefore, this work firstly demonstrated that the surface proteins of HC-2 played an important role in the process of probiotic-function that regulation of immune response, adhesion and colonization,

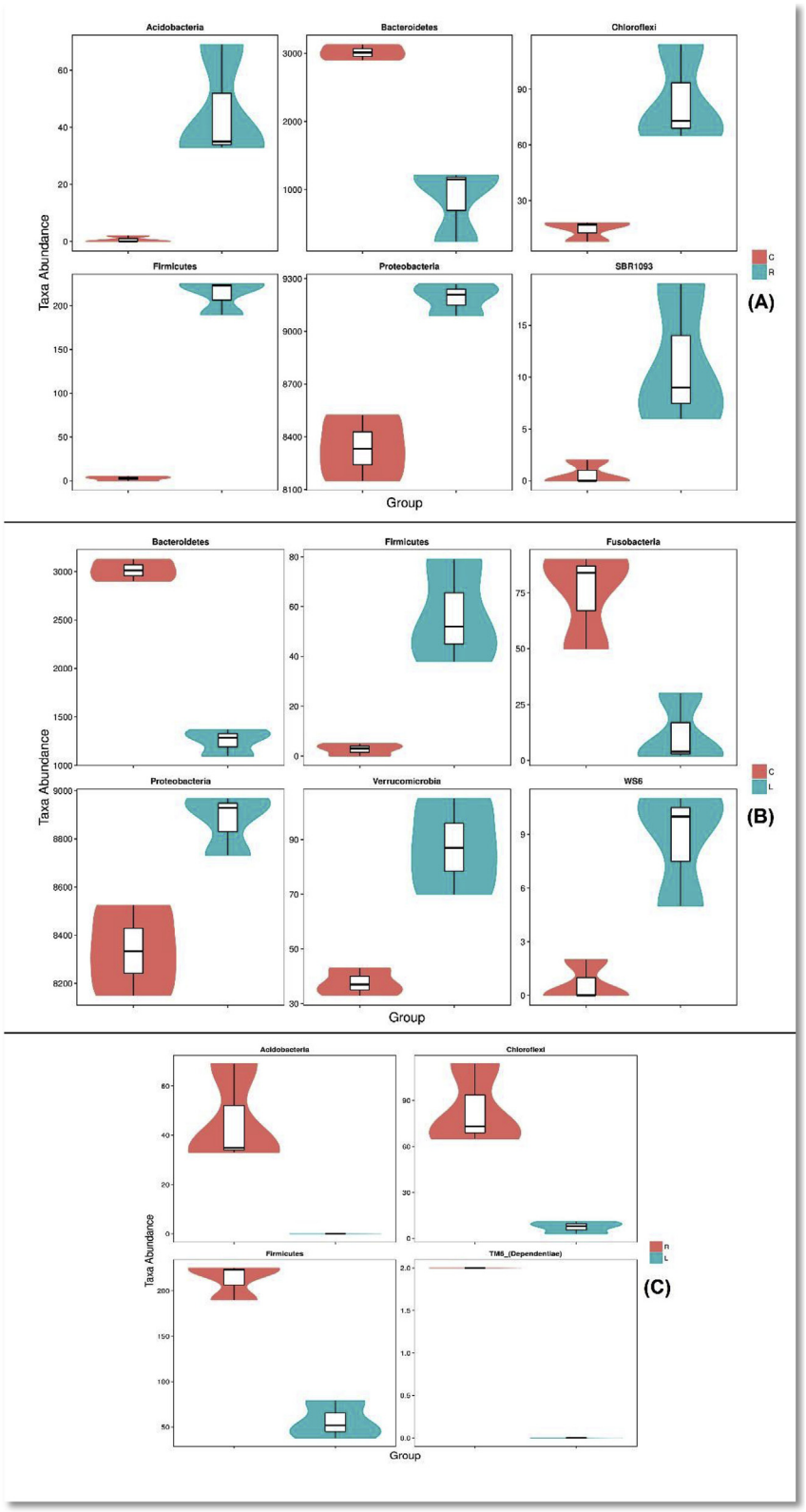


Fig. 8. The significantly different phyla in three treatments analyzed by Metastats. Shrimps were fed a basal diet (C) or a basal diet supplemented with *L. pentosus* HC-2 (R), LiCl-treated *L. pentosus HC-2* (L).

competitively exclusion of pathogens, promoting beneficial bacteria and so on. Whereas, the mechanism was blocking the adhesion or affect the metabolism of bacteria after the surface proteins "shaving off" of HC-2 thus lead to the bacteria composition varied was not very clear, and also needed be further research.

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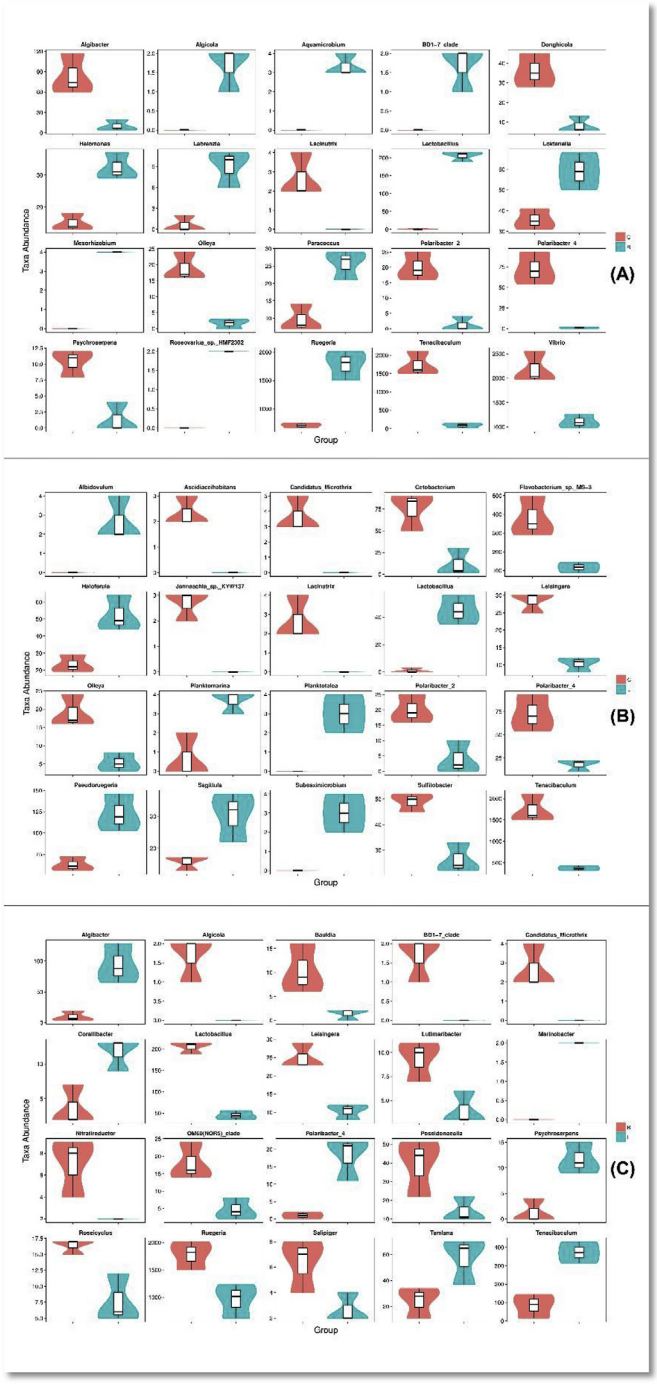

Fig. 9. The top twenty significant different genus among three treatment groups analyzed by Metastats. Shrimps were fed a basal diet (C) or a basal diet supplemented with *L. pentosus* HC-2 (R), LiCl-treated *L. pentosus* HC-2 (L).

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