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Short communication

The influence of surface proteins on the probiotic effects of *Lactobacillus pentosus* HC-2 in the *Litopenaeus vannamei* hepatopancreas

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ABSTRACT

Our previous work showed that *Lactobacillus pentosus* HC-2 has high antibacterial and adhesion activity, and as a probiotic could improve the nutrients and immunomodulatory effects in the *Litopenaeus vannamei* farming. In order to further investigate the influence of HC-2 surface protein on its probiotic effects, the immune and digestion related genes expression and enzymes activities, the colonization numbers of HC-2, and the histologic characteristics were analysis in shrimp hepatopancreas after feeding either the intact surface proteins, the probiotic treated with lithium chloride (LiCl) to remove noncovalently bound surface proteins or no probiotic for four weeks. The results showed that the immune genes expression of lysozyme, proPO, LGBP, Penaeidins-3α, crustin and C-type lectin, the immune enzymes activities of superoxide dismutase, catalase and Alkaline phosphatase, and the digestion enzymes of Trypsin, Lipase and α-Amylase were significantly higher in hepatopancreas of shrimp fed with intact HC-2 than that in shrimp fed with base diet or striped surface proteins HC-2 post feeding and challenge. In addition, the shrimp fed with intact HC-2 leads to the bacteria cells adhesion to hepatopancreas was significantly higher than that in shrimp fed with no surface proteins HC-2. Furthermore, the tissue damages of hepatopancreas caused by pathogenic vibrio were obviously observed in shrimp fed with base diet or no surface proteins HC-2, but no signs of damages were found in shrimp fed with intact HC-2. These results demonstrate that surface proteins are important components for HC-2 to execute probiotic effect that improve hepatopancreas immune response and nutrition digestion to protect shrimp against pathogen damage.

1. Introduction

Probiotic, a beneficial microorganism, which has been used as one of the alternative antibiotic, can confer favorable effects such as better resistance to pathogens, enhancing growth and immune stimulation of the host organism when used in adequate levels [1]. Initially, probiotics were used as animal feed additives in cattle and pigs because of their beneficial effects, including acting antagonistically against pathogenic microorganisms, competing for adhesion sites and nutrients with pathogens, and modifying the host immune response [2,3]. In recent years, probiotics have been widely used in aquaculture to defense against intestinal diseases and improve growth performance of cultured animals [4,5]. Among the available probiotics, lactic acid bacteria (LAB) are commonly used and advocated. LAB, a group of Gram-positives, rod- or coccus-shaped, generally non-motile, non-cytochromes, and non-sporulating bacteria, which produce lactic acid as a major or sole product from the fermentation of glucose [6]. They are a common native group in gastrointestinal tract of various animals and some are increasingly accepted as predominant probiotics [4,7]. The preservative effect of lactic acid bacteria is mainly including improving the immune status, modulating the bacterial community and the acidic conditions that they create which inhibit the growth of opportunistic pathogens [8–10].

For the probiotics, adhesion to the intestinal mucosa epithelium or mucus is the prerequisite exhibiting documented health effects, and the surface proteins have been proposed to be involved in colonization [11]. Indeed, except for mediating binding ability, surface proteins are also involved in maintaining the shape of the bacteria, functioning as molecular sieves, providing immunomodulation and extracellular enzyme binding sites to the host [12,13]. As known, the hepatopancreas is recognized as the main storage and immune organs in shrimp, which can perform digestion and absorption, energy storage, detoxification,

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Table 1
Primers used in this study.

Target gene	Sequence (5' - 3′)	Product size (bp)	Accession NO.
pentF pREV	F-CAGTGGCGCGGTTGATATC R-TCGGGATTACCAAACATCAC	220	AJ292254
β-Actin	F-GCCCATCTACGAGGGATA R-GGTGGTCGTGAAGGTGTAA	121	JF288784
Lysozyme	F-TCTGCAAAATCCCATGTTCCG R-AGTTGGAACCGTGAGACCAG	198	AY170126
Crustin	F-CGGAGTAGGTGTTGGTGGTGGTT R-CTCGCAGCAGTAGGCTTGAC	178	AY486426
ProPO	F-CGGTGACAAAGTTCCTCTTC R-GCAGGTCGCCGTAGTAAG	120	EU284136
Penaeidins-3α	F-CTCGTGGTCTGCCTGGTCTTCTTG R-CAGGGCAACCGTTGTATGGA	151	FJ472345
C-type lectin	F-GATCGAGGACTGCGAAACCT R-CCCCAGAAAGGTACACCCTG	162	EF583939
LGBP	F-TGGACGCTTATGTCACCTAC R-CTTCTACTTCATCTGTTGCT	177	JN415536

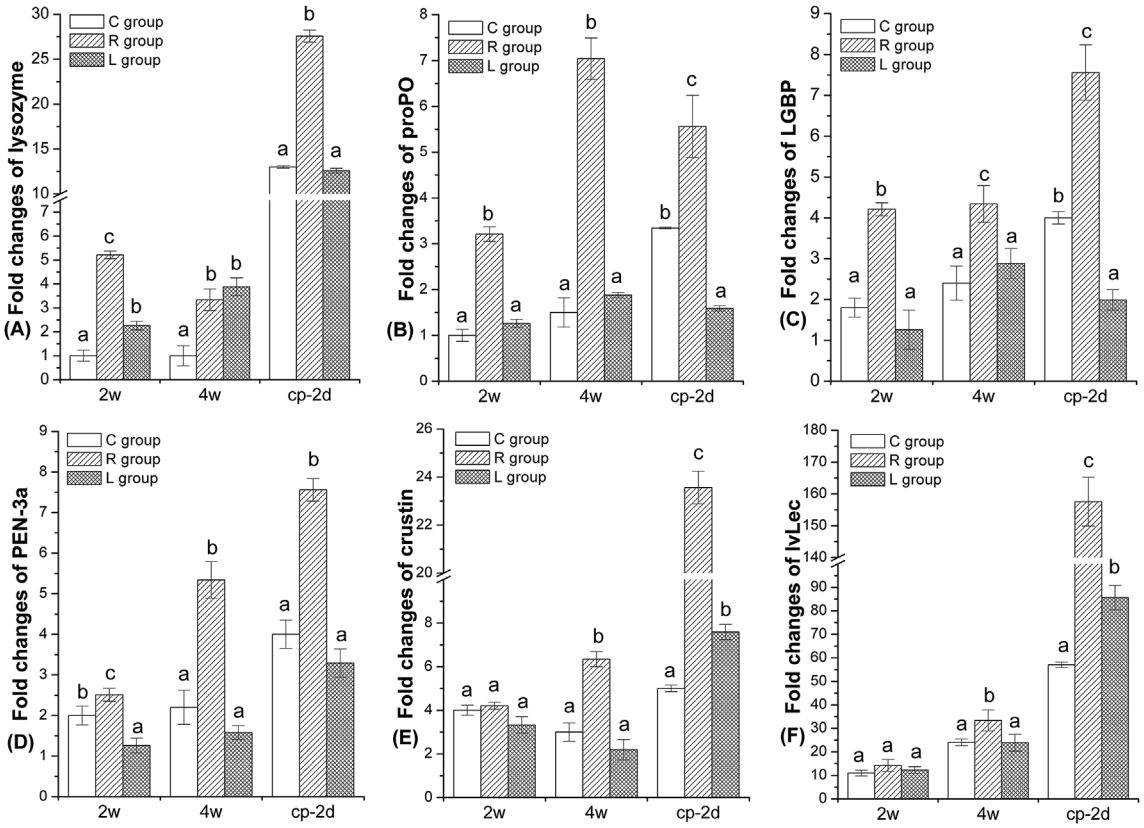

Fig. 1. Relative expression of the immune-related genes (lysozyme, proPO, LGBP, PEN-3α, crustin, and lvLec) in the *L. vannamei* hepatopancreas at two and four weeks post-feeding, and at two days after challenge (cp-2d) were measured by SYBR green qPCR. The letters above the bars that are not the same are significantly different at $P < 0.05$ based on ANOVA. Note: C, shrimp were fed a basal diet; R, shrimp were fed a basal diet supplemented with normal *L. pentosus* HC-2; L, shrimp were fed a basal diet supplemented with LiCl-treated *L. pentosus* HC-2.

hematopoiesis and osmotic pressure regulation [14]. Several studies reported that probiotic could improve the resistance of prawns to diseases by regulating the immune of hepatopancreas [15,16]. Our previous work found that *Lactobacillus pentosus* HC-2 has perfect probiotic to *Litopenaeus vannamei* [17,18], and the surface proteins of HC-2 play a vital role in mediating the bacteria to improve shrimp intestine immune response, bacterial diversity and disease resistance [19]. However, the effects of surface proteins of HC-2 on its probiotic in the *L. vannamei* hepatopancreas has not been characterized.

2. Materials and methods

2.1. Ethnic statement

This study was conducted strictly in line with the procedures in the Guide for the Use of Experimental Animals of the Institute of Oceanology, Chinese Academy of Sciences. In this study, for the methods used in animal experiments were approved by the Institutional Animal Care and Use Committee of Institute of Oceanology, Chinese Academy of Sciences (Permit Number: HAIFAJIZI-2013-3). All efforts were dedicated to minimize suffering.

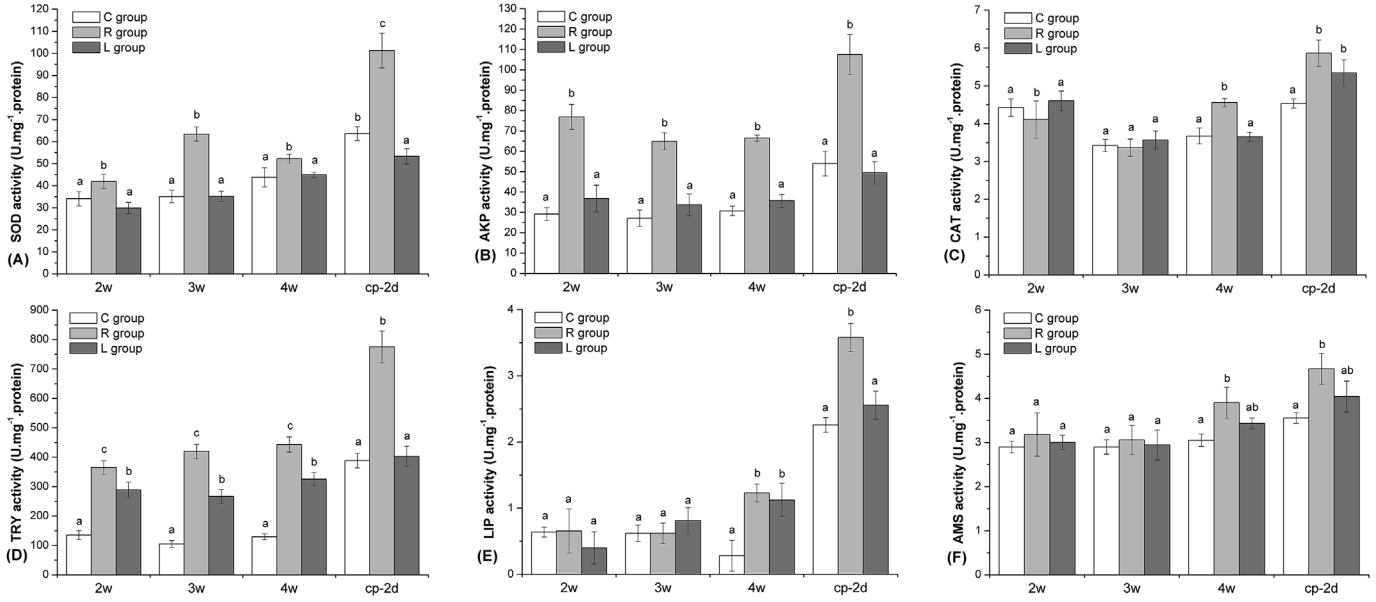

Fig. 2. Immune and digestion related enzyme activities in hepatopancreas of *L. vannamei* at one to four weeks post feeding, and at two days after challenge (cp-2d). A–F: (Superoxide Dismutase, SOD), (Alkaline phosphatase, AKP), (Catalase, CAT), (Trypsin, TRY), (Lipase, LIP), (α-Amylase, AMS). Mean values and standard deviation (± SD) are presented for each parameter (n = 10). Means in the same row with different letters are significantly different ($P < 0.05$). Shrimp were fed a basic commercial diet (C), or supplemented with *L. pentosus* HC-2 (R), or supplemented with LiCl-treated *L. pentosus* HC-2 (L).

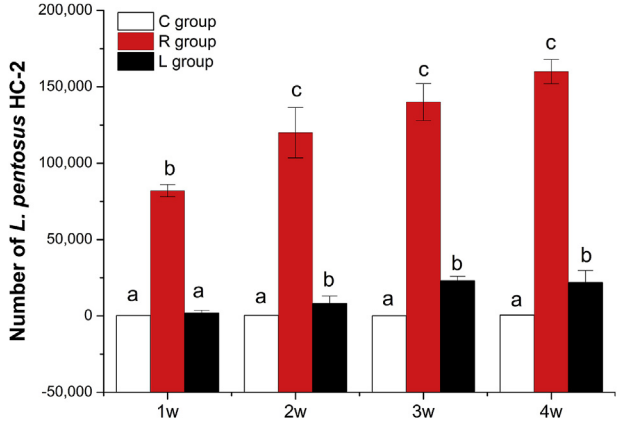

Fig. 3. Measurement of the colonization numbers of *L. pentosus* HC-2 during the experimental feeding in hepatopancreas of shrimps. After taken the total DNA of the tissues, the specific RecA primer was used for real-time PCR. The number of HC-2 were calculated according to equation $Y = -3.1154x + 33.651$ ($R^2 = 0.9907$). Values were means ± SE, and different letters denoted significant difference ($P < 0.05$). Shrimp were fed a basal diet (C) or a basal diet supplemented with *L. pentosus* HC-2 (R), LiCl-treated *L. pentosus* HC-2 (L).

2.2. Bacterial growth and surface protein shaving

Bacterial culture and cell surface protein shaving was performed as previously described [19]. Briefly, *Lactobacillus pentosus* HC-2 was grown in MRS broth at 37 °C under anaerobic conditions for 18 h. A 500 mL culture of bacteria on the transition between late exponential and stationary phase ($OD_{600} \approx 1.7$) were harvested by centrifugation (3000 × *g*, 10 min, 4 °C). Then, the cell pellets were washed three times with 1 M phosphate-buffered saline (PBS) containing 25% sucrose. After centrifugation, the bacterial cells were incubated in 25 mL of 5 M LiCl to strip the surface-associated proteins. After treatments, the cells were collected and washed three times with autoclave sterilized seawater.

2.3. Feeding trials

The experimental diets were prepared as previously described [19]: bacteria were resuspended in sterilized seawater and sprayed on basal commercial feed (containing crude protein 42%, crude fat 7%, ash 15%, and water 11%) at 5×10^8 colony-forming units (CFU) g/feed. Experimental shrimp (3.0 ± 0.20 g) were obtained from Ruizi Seafood Development Co. Ltd. in Qingdao (Shandong, China). A total of 900 healthy shrimp were randomly divided into 9 aquaria (600 L), each aquaria containing 100 shrimp. Three groups: C group, shrimp fed with the commercial basic diet; R group, shrimp fed with the commercial basic diet + HC-2; L group, shrimp fed with the commercial basic diet + LiCl-treated HC-2, and each diet treatment was conducted with triplicate experiments. Shrimp were maintained in fresh seawater (salinity, 30‰; temperature, 30 ± 2 °C) with continuous aeration. During the experiment period, shrimp were fed three times per day (at 7:00, 11:00 and 19:00) for 4 consecutive weeks, and daily feeding rate was 10% of the body weight.

2.4. Challenge assay

Challenge test was performed as previous work [19], a pathogenic strain of *Vibrio parahaemolyticus* E1 was 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 culture conditions were the same as previously mentioned.

2.5. Changes in immune gene expression

To investigate the effects of experimental diets on the immunity of *L. vannamei*, the hepatopancreas from 10 shrimp per treatment were sampled at 2, 3, 4 weeks post-feeding, and at two days after challenge. RNA was extracted from tissues using an RNA fast extraction kit according to the manufacturer's protocol (Fastagen, China) and the RNA concentration and integrity were measured using NanoDrop ND-1000 (NanoDrop Technologies, USA). A total of approximately 2 μg of RNA was used for cDNA synthesis using a TransScript® One-Step gDNA

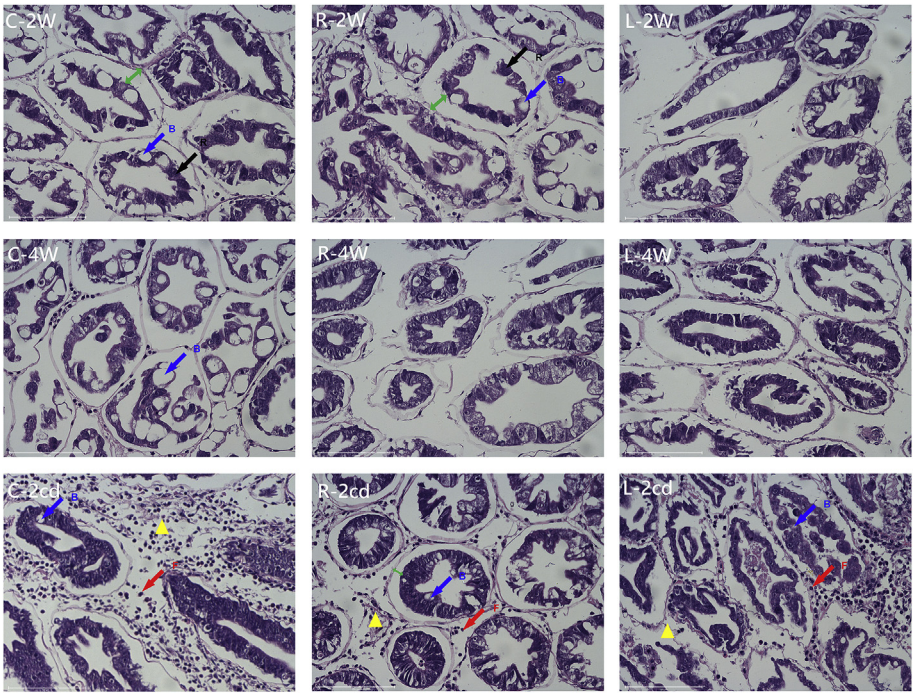

Fig. 4. Histology with hematoxylin and eosin staining of the shrimp hepatopancreas at two and four weeks post-feeding, and at two days (cp2d) post challenged by *Vibrio parahaemolyticus* E1. Images are arbitrarily chosen examples of the histology observed in three groups. Double arrow heads indicate separation between the myoepithelial layer and the epithelium. Triangle indicate multifocal necrosis with loss of tissue. Blue arrow indicated B-cell (secretory cells), black arrow indicated R-cell (Reduction of storage cells), and red arrow indicated F (fat globule). The magnification is 40×, and the scale is 100 μm. Shrimp were fed a basal diet (C) or a basal diet supplemented with *L. pentosus* HC-2 (R), LiCl-treated *L. pentosus* HC-2 (L). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Removal and cDNA Synthesis Kit according to the manufacturer's protocol (TransGen Biotech Co., Ltd., China).

The expression profiles of six immune-related genes of lysozyme, proPO, LGBP, Penaeidins-3α (PEN-3α), crustin, and C-type lectin (lvLec) were investigated using a Roche480 real-time PCR system (LightCycler 480, USA). *β*-Actin was selected as the internal control for RT-PCR. The cloned amplicons were confirmed by sequencing. 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 analysed with Microsoft Excel, and the relative expression ratio (R) of mRNA was calculated according to $2^{-\Delta\Delta Ct}$ method.

2.6. Enzyme activity detection

At 2, 3, 4 weeks post-feeding, and at two days after challenge, 10 shrimps were randomly selected from each aquariums, then sampled the hepatopancreas and stored in liquid nitrogen. Following, the tissues were homogenized 1:10 w/v in ice-cold 50 mM Tris-HCl buffer (pH 7.5), centrifuged at 12,000 g for 15 min at 4 °C, and the supernatant was taken for evaluation of enzymatic activity. The activity of antioxidant enzymes superoxide dismutase (SOD), catalase (CAT) and Alkaline phosphatase (AKP), and digestion enzymes of Trypsin (TRY), Lipase (LIP) and α-Amylase (AMS) were measured using commercial colorimetric kits (Nanjing Jiancheng Bioengineering Research Institute) according to the operation manual [20–22].

2.7. Measurement of the colonization numbers of L. pentosus HC-2

The numbers of *L. pentosus* HC-2 were detected as previous report [19]. The hepatopancreas of 10 shrimp of each group were sampled at one to four weeks post feeding experiment, and the total DNA were extracted using DNeasy Blood & Tissue Kit (Qiagen, Germany). RNase free water was used as negative control, and each dilution with triplicate reaction. The specific RecA primer was applied to amplify the PCR reaction. A total reaction volumes of 20 μl containing 2 μl 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. After the Ct values were obtained, the number of *L. pentosus* HC-2 were calculated according to equation $Y = -3.1154x + 33.651$ ($R^2 = 0.9907$).

2.8. Histopathology

The histology was performed as described by Sha et al. (2016) [18]. At 2, 4 weeks post-feeding, and at two days after challenge, the hepatopancreas of 6 shrimp of each group were sampled for fixation (60% absolute ethanol, 30% trichloromethane, 10% acetic acid) for 19 h. Then, the fixed tissues were dehydrated in ascending concentrations of alcohol (70, 80, 95, and 100%), cleared in toluene, and embedded in paraffin. Tissue sections (0.5 mm thick) were obtained with a rotary microtome. Tissue sections were stained with hematoxylin and eosin, and histopathological changes analysed microscopically (Olympus CKX41 microscope, Tokyo, Japan).

2.9. Statistical analysis

The statistical analysis was performed using Sigma Stat software (SPSS Inc. version 19.0), One-way ANOVA and Duncan's multiple comparisons of the means were done to compare data obtained. All graphics rendering were generated with origin 8.0 software, the results are expressed as mean ± standard error (SEM) and the differences were considered significant level at $P < 0.05$.

3. Results and discussion

In shrimp, the hepatopancreas is the main organ of the immune and digestive system, which has functions including synthesis and secretion of digestive enzymes and of immune molecules, to the absorption, storage and excretion of nutrients [23]. The antimicrobial peptides (AMPs) of penaeidin, crustin and lysozyme, the proPO system and the clotting cascade are the critical components of the innate immune systemin invertebrates, which play prominent roles in host defence against pathogens [24,25]. In the present study, the selected immune related genes were increased significantly higher in shrimp fed with

intact HC-2 than in shrimp fed with base diet or with striped surface proteins HC-2 at two and four weeks post-feeding. However, these genes were expressed no difference between C group and L group (Fig. 1A–F). After the experimental shrimp were challenged by *V. parahaemolyticus* E1, these genes were induced significantly higher up-regulation in R group than that in C group or in L group. Except the crustin and lvLec were induced significantly higher expression in L group than that in C group (Fig. 1E and F), the other genes transcript level were displayed no difference between C group and L group. This results demonstrate that surface proteins play vital roles in mediation HC-2 improve the shrimp hepatopancreas immune response.

Production of reactive oxygen species (ROS) is a vital defense mechanism of crustaceans against pathogens, however, over-production of ROS and residual ROS can cause serious damage to cells and tissues. SOD, AKP and CAT were important antioxidant enzymes, which can maintain the redox balance, and prevent, or repair oxidative damage [26]. The feeding experimental results showed that the activities of SOD, AKP and CAT were increased significantly higher in R group shrimp than that in C group or L group from two weeks to four weeks post feeding and challenge (Fig. 2A-C), which suggest that supplement with intact probiotic can improve shrimp oxidation resistance to against pathogens. Digestive enzyme levels was usually used for evaluating the assimilation of nutrition, many studies have reported that diet with probiotics increased the trypsin, lipase and α-amylase in shrimp [27,28]. The present study observed that the activities of trypsin had a significantly increasing in R group than that in C group or L group from two to four weeks post feeding and at two days after challenge, and the L group showed significantly higher than C group from two to four weeks post feeding, but the activity showed no difference between L group and C group after challenge (Fig. 2D). For lipase and α-amylase, the activities were only showed significantly difference at four weeks post feeding and at two days after challenge. R group also showed significantly higher than C group or L group (Fig. 2E and F. This results indicated that surface proteins were important factor for the HC-2 to regulate the digestion in hepatopancreas of shrimp.

Whether lactobacillus can colonize and develop into dominant flora in animal intestinal tract is the precondition for its probiotic function. The previous work had detected that the surface proteins affected HC-2 adhesion to shrimp midgut [19], similar results observed in shrimp hepatopancreas in present study that the colonization numbers of HC-2 were increasing significantly higher in R group than that in C group or L group from one week to four weeks post feeding, however, the colonization numbers of HC-2 were increased stably at two weeks (Fig. 3). This results suggest that surface proteins are essential components for HC-2 to adhesion to hepatopancreas of shrimp to enhance the immune response and the digestion capacity.

There was no significant difference observed from the hepatopancreatine histology at two weeks and four weeks post feeding with intact HC-2 or striped surface proteins HC-2 compared with shrimp fed with base diet. However, after the challenge, obvious histological alterations caused by *V. parahaemolyticus* E1 was observed in the hepatopancreas of C group and L group shrimp. As shown in Fig. 4, the myoepithelial layer were separated from the epithelium of the hepatopancreas, the numbers of R-cells decreased markedly, and star-shaped polygonal structures of the lumen disappeared in some individuals. In addition, there was excess fat globule in many vacuolated cells, nuclear pyknosis, cell necrosis and cell lysis. Whereas, the damages caused by vibrio was not obvious in R group shrimp hepatopancreas. This results indicate that surface proteins were important for HC-2 to protect shrimp hepatopancreas against cell and tissue damages caused by pathogens.

In conclusion, in present study, the probiotic *L. pentosus* HC-2 with intact surface proteins significantly induced higher immune genes expression, and increased the immune enzymes activities and digestion enzymes level, and increased the number of bacterial colonization in shrimp hepatopancreas, and protect the hepatopancreas from pathogens damage. These findings are consistent with previous studies of challenge assay that intact surface proteins probiotic supplemental shrimp obtained a better immune protection effect than the control group or the no surface proteins probiotic supplemental group [19]. The work demonstrate that surface proteins are important components for HC-2 to execute probiotic effect, which are essential for the bacteria colonization to improve hepatopancreas immune response and nutrition digestion to protect shrimp against pathogen's infection. The findings in present work will help to understand the probiotic mechanism of lactobacillus on shrimp.

Acknowledgements

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