



Full length article

Screening of intestinal probiotics and the effects of feeding probiotics on the growth, immune, digestive enzyme activity and intestinal flora of *Litopenaeus vannamei*

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ABSTRACT

In this experiment, 426 strains were isolated from the intestinal tract of *Litopenaeus vannamei*, and 11 strains showed strong digestive enzyme production activity and antagonistic effect against common bacterial pathogens of shrimp. After hemolysis activity test and drug sensitivity test, 2 candidate probiotics with good bacteriostatic activity, strong enzyme production ability and relatively sensitive to antibiotics were screened out, and were identified by 16s rDNA molecular identification and Biolog-System as *Enterobacter hominis* (E3) and *Lactobacillus* (L3). First, the biological characteristics of 2 candidate probiotics were studied. The optimum growth conditions of E3: temperature, 30 °C; pH, 8.0; NaCl, 2.5%; bovine bile salt, 0.15%; and the optimum growth conditions of L3: temperature, 40 °C; pH, 6.0; NaCl, 0.5%; bovine bile salt, 0.0015%. Secondly, a 28-day feeding experiment was conducted using probiotic concentration of 10^7 CFU g⁻¹ to determine the changes of the activities of blood related immune enzymes (SOD, PPO, ACP, POD, CAT, LZM) and intestinal digestive enzymes (NP, AL, LPS) during the feeding process of shrimp, the results showed that during the course of feeding, the activities of immune enzyme and digestive enzyme of shrimp fed with probiotics showed an increasing trend, and the growth rate of body weight of shrimp was higher than that of control group. After feeding, the cumulative mortality of probiotics groups were significantly lower than that of the control group after WSSV infection. And the mid-gut of *L. vannamei* was observed by electron microscope, the results showed that the intestinal mucosa was tight and the epithelium cells showed an active secretory state in probiotics group. Finally, the intestinal microbial communities of shrimp were compared and analyzed by using Biolog-ECO method in the later period of feeding, the results showed: compared with the control group, the average color change rate of the experimental group fed with probiotics increased significantly, indicating that probiotics enhanced the intestinal microorganism activity; The ability of intestinal microorganism to utilize carbon source was significantly enhanced in the experimental group, which indicated that the digestive enzyme secreted by probiotics could improve the digestion and absorption rate of prawn feed, thus promoting the rapid growth of shrimp; The Shannon index, Simpson index and McIntosh index of probiotics groups showed significant difference in 1st and 5th days, but tended to be the same in the 10th day, the results showed that probiotics could maintain in *L. vannamei* intestines at least 5 days.

1. Introduction

With the rapid development of shrimp culture, the diseases of shrimp culture have become increasingly prominent, and the increasingly serious environmental problems have been threatening the shrimp culture [1–3]. Studies have shown that the use of a large number of antibiotics not only disturbs the normal flora of the intestinal tract of

shrimp and makes the pathogens resistant to drugs, but also the residue of the drug in the environment and in the prawn body ultimately threatens the health and safety of human [4,5].

In view of the fact that probiotics do not produce residues or drug resistance in animals, probiotics as a substitute for antibiotics have become a hot topic of research. Many studies have shown that supplemental probiotics can not only improve the survival and growth rate

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of aquatic animals, but also reduce the outbreak of diseases by improving the immune system of fish and shrimp [6]. Probiotics have been widely used in aquaculture as an effective way to control diseases, improve immunity, provide nutrition, promote digestion and control water quality [7,8].

The isolation of strains with various digestive enzyme activities from the intestinal tract of healthy animals has become one of the effective ways to screen probiotics [9,10]. It has been shown that the selected beneficial bacteria should be used in the shrimp body, which can not only improve the utilization rate of the shrimp feed and promote the growth of the shrimp, some probiotics can but also promote the immunity of shrimp immune system to virus and pathogenic bacteria and reduce the outbreak of shrimp disease [11].

In this experiment, the candidate probiotics were screened from the intestinal tract of healthy shrimp and feeding experiment was conducted to attempt to evaluate the biological function of the intestinal endogenous probiotics.

2. Materials and methods

2.1. Experimental animals

Experimental *Litopenaeus vannamei* were obtained from a local farm in Dagang, Tianjin, China and temporary cultured in the laboratory for two weeks before feeding experimentations.

2.2. Reagents and materials

MRS medium, YPD medium, TSB medium, LBs medium, 2216E medium, and bacillus isolation medium were purchased from Luqiao Technology Co., Ltd (China). Various enzyme activity kits were purchased from Suzhou Keming Biotechnology Co., Ltd (China). PCR primers were synthesized from Sangon biological engineering Co., Ltd (China). Biolog Microstation instruments and related identification reagents were purchased from Guangdong Huayue instruments Co., Ltd (USA).

2.3. Screening and identification of intestinal probiotics in healthy *L. vannamei*

2.3.1. Screening of strains with strong enzyme production ability and antimicrobial activity

After 75% alcohol disinfection of healthy *L. vannamei*, the intestines were ground in homogenizer under aseptic condition, and the grinding fluid was diluted with NSS [12]. The grinding fluid of different parts and different dilutions were coated on the MRS medium, YPD medium, TSB medium, LBs medium and 2216E medium. The isolation of *Bacillus* needs enrichment culture, and then coated on the isolation medium of *Bacillus*, which is cultured at constant temperature of 28 °C for 24–48 h. The single colonies were inoculated and purified on the corresponding culture medium at least 3 times. The lipase, amylase and protease activities and the common antibiotic susceptibility test of the purified strains were detected, and antagonistic experiment was carried out with common pathogens in shrimp diseases by filter paper spot method on culture medium [13].

2.3.2. Species identification of strains

The strains were identified by molecular analysis, the genomic DNA was extracted and 16S rDNA was amplified by primers 27F (5'-AGAG TTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTGTACGA CTT-3'). The reaction conditions of PCR: 94 °C for 4 min followed by 30 cycles of 94 °C for 30 s, 50 °C for 1 min and 72 °C for 2 min, and 72 °C for 10 min. The amplified products (about 1.5kp) were sequenced (commissioned by Beijing Yingweijieji Technology Co.Ltd.) and the 16S rDNA sequence was compared and identified in the GenBank database (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). Further species identification

was carried out by using Biolog-System microorganism identification method.

2.3.3. Biological characteristics of strains

According to the experimental methods of Kaynar, the single colony of the candidate probiotics were selected for overnight culture on the corresponding culture medium, and the heavy suspension solution was cultured under different culture conditions [14]. The optimum growth conditions of the strain were investigated, including temperature, pH, the concentration of NaCl and bovine bile salt.

2.4. Feeding experiment

2.4.1. Feeding method

The experiment was divided into three groups, of which group E3 which added *Enterobacter hormaechei* and group L3 which added *Lactobacillus* in the feed, while the blank group was the same feed with equal amount of PBS, triplicate (three aquaculture cylinders) in each group. A total of 250 shrimps with body length of 4 cm were placed in each pond. The shrimps were fed with 5% of the body weight of the shrimps twice a day. The concentration of bacteria in the feed was 10^7 CFU g⁻¹, and half water was changed daily and fed 28-day in this way [15–17]. Water quality was strictly controlled to eliminate interference: ammonia, 0.03–0.06 mg mL⁻¹; nitrite, 0.013–0.019 mg mL⁻¹; temperature, 28–33 °C; pH, 8.0–8.5; salinity, 18–21‰.

2.4.2. Determination of blood-related immune enzyme and intestinal digestive enzyme activity

Sampling was conducted every 7 days during feeding, and 20 shrimps were randomly selected from each group. Blood cells and serum were obtained by centrifugation under aseptic condition at 4 °C, 800 g for 10 min. The blood cells were stored in 1 mL Trizol at –80 °C, and the serum was preserved at 4 °C. The activities of superoxide dismutase, polyphenol oxidase, acid phosphatase, lysozyme, peroxidase and catalase in blood were determined according to the requirements of the kit. At the same time, the intestinal tract of shrimps were ground with buffer solution in the kit to prepare the sample, and the activities of protease, amylase and lipase in the intestinal tract were determined according to the requirements of the kit.

2.4.3. Determination of *L. vannamei* growth

Before the experiment began, 10 shrimp s were randomly collected from each group of ponds, their body weight (W_0) were measured. During feeding, 10 shrimps were randomly selected from each group to measure their body weight (W_t) every week. The weight gain rate of each group of shrimps were calculated:

$$\text{Weight gain rate (\%)} = \frac{W_t(\text{g}) - W_0(\text{g})}{W_0(\text{g})} \times 100\%$$

2.4.4. Determination of cumulative mortality of *L. vannamei* after WSSV infection

After feeding, 120 shrimps were collected from each group, and each group included three replicates. The shrimp was injected intramuscularly with the prepared WSSV particle suspension and 10 µl was injected into the second abdominal somite of shrimps. The cumulative mortality of shrimp was recorded every 12 h to evaluate the effect of probiotics on the resistance of shrimp after the muscle-injection of WSSV.

2.4.5. Electron microscopic observation of mid-guts of *L. vannamei*

After feeding, 10 shrimps were collected randomly from each group, and guts were sliced with sharp blades with a 2.5% glutaraldehyde solution, rinse out contents several times with 4% glutaraldehyde solution. Specimens were dehydrated sequentially in 60%, 80%, and 100%

alcohol for 15, 30, and 45 min, respectively. Obtained samples were observed under a scanning electron microscope (SEM; Hitachi TM-1000, Japan) at 15 kV and a transmission microscope (TEM; Hitachi TEM-1200, Japan) at 80 kV.

2.4.6. Detection of intestinal microbial community of shrimp by Biolog-ECO method

After the 28-day feeding test, 20 shrimps were randomly selected from the experimental group and the control group at the 1, 5, 10 day to test the intestinal microbial community. 75% of alcohol surface disinfectant was cleaned with 0.8% of normal saline, dissected in sterile state, intestinal tract was removed, and was homogenized with physiological saline in ice bath of homogenizer. The final volume of homogenate was 20 mL. Shake the homogenate into the Biolog-ECO microplate [18], add 150 μ L to each hole, triplicate. The microplate is cultured in 30 °C for 4 h and then was read by Biolog-System readout every 24 h till 5 days.

2.5. Statistical analysis

Results are presented as means \pm standard deviation from triplicate experiments. The data were analyzed for significance by one-way ANOVA and then followed by Duncan multiple comparison tests using SPSS Software version 11.0. P value less than 0.05 was considered statistically significant.

3. Results

3.1. Candidate probiotics

A total of 426 strains from the gut of *L.vannamei* were screened on 6 kinds of culture medium, among which 11 strains had enzyme production and bacteriostatic effect (Fig. 1). Then the hemolytic test and antibiotic susceptibility test were carried out on these 11 strains to determine the safety of the strains. The results showed that none of the 11 strains show hemolytic activity, and 3 strains were resistant to common antibiotics, the other were sensitive to many kinds of antibiotics (Table 1). According to the results of bacteriostasis activity test, digestive enzyme activity test and safety test, 2 sensitively probiotics strains that had strong enzyme production and bacteriostatic effect were screened and were identified as *Enterobacter hormaechei* (E3) and *Lactobacillus* (L3) by 16S rDNA molecular identification, and the homology ratio was 100% and 99.9% respectively in the GenBank database. Then Biolog-System was used to identify again, further confirmed the identification results.

3.2. Biological characteristics of candidate probiotics

Enterobacter hormaechei (E3), Gram-negative bacteria, facultative anaerobic, peritrichate motility, can grow widely. The optimum growth conditions of strain E3: temperature, 30 °C; pH, 8.0; NaCl

concentration 2.5% and bovine bile salt concentration 0.15% (Fig. 2); *Lactobacillus* (L3), Gram-positive bacteria, facultative anaerobic, the optimum growth conditions of L3: temperature, 40 °C; pH, 6.0; NaCl concentration 0.5% and bovine bile salt concentration 0.0015% (Fig. 2).

3.3. Blood-related immune enzyme and intestinal digestive enzyme activity

The activities of PPO, SOD, ACP, POD and CAT in the blood of *L.vannamei* fed with probiotic E3 were significantly ($p < 0.05$) increased during the whole feeding process, except the LZM activity decreased on the 14th day. The activity of the same enzymes in the blood of probiotic L3 showed the same tendency of E3 except slightly lower than that in the E3 group. The PPO activity of L3 group decreased on the 7th days (Fig. 3). The activities of neutral protease and lipase in the intestinal tract of *L.vannamei* after feeding probiotic bacteria were significantly increased ($p < 0.05$), but on the whole, the activity in group L3 was lower than that in group E3, and the activity of amylase showed significant difference after feeding probiotics for four weeks, compared with the blank group (Fig. 4).

3.4. Growth rate of *L.vannamei*

During the period of shrimp culture and feeding, there was no significant ($p < 0.05$) difference in the first week; at the second week, the L3 group (8.95%) was slightly higher than the E3 group (7.05%) and the blank group (6.98%); at the third week, the L3 group (10.94%) and E3 group (10.79%) were significantly higher than the blank group (8.25%). After the end of feeding, the probiotics groups were significantly higher than the control group (10.05%), and the effect of E3 group (13.75%) was more significant.

3.5. Cumulative survival rate of *L.vannamei* after WSSV infection

After WSSV infection, the cumulative mortality rates of control group and probiotics groups were almost the same at 24 h, and there's a minor difference at 36 h. The cumulative mortality of shrimp in blank group was significantly higher than that in experimental group at different time points after 48 h, among which, the cumulative mortality rate in E3 group was always lower than that in L3 group (Fig. 5).

3.6. Electron microscopic observation of mid-gut of *L.vannamei*

The results of electron microscopic scanning showed that there were significant differences in the inner surface of intestinal tissues in different samples. The density and fold depth of probiotics group were higher than that of the control group, and there were a series of small bulges on the surface. In addition, the fold density of E3 group was higher than that of L3 group, and there were many clusters in L3 group. The results of transmission electron microscope showed that the integrity of epithelial cells and the density of mucosal layer in probiotics

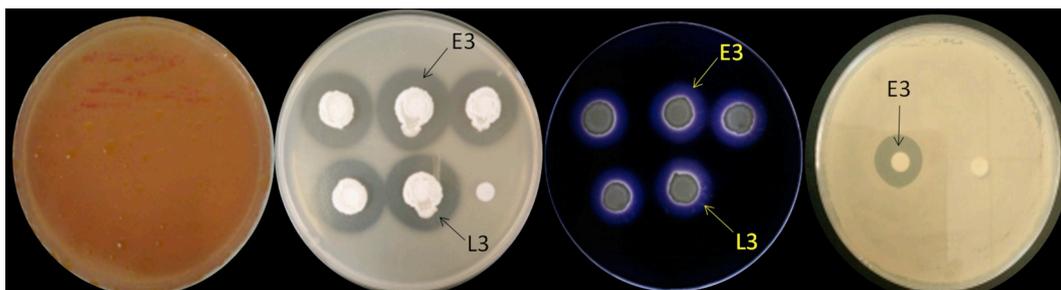


Fig. 1. Experimental positive results of three digestive enzymes and antagonistic spot. (A) Protase positive of E3; (B) amylase positive; (C) lipase positive; (D) antagonistic spot of E3.

Table 1
Susceptibility of 11 strains to common antibiotics (Notes: R. resistance; I. intermediate; S. sensitive.).

antibiotics	Susceptibility of candidate probiotic strains										
	B1	B2	E3	B3	B4	L3	B5	E4	B6	B7	B8
Erythromycin	R	I	R	S	R	S	R	R	S	R	S
Midecamycin	S	I	R	R	R	S	S	R	S	R	S
Tetracycline	R	S	S	I	R	S	I	I	R	S	I
Lomefloxacin	S	R	S	R	S	R	R	S	R	R	S
Ciprofloxacin	S	R	S	R	R	S	I	S	R	R	S
Chloramphenicol	R	S	S	S	S	S	S	R	R	S	I
Sulfamethoxazole	I	R	S	S	S	S	R	R	R	R	I
Nitrofurantoin	R	R	R	R	R	R	R	R	R	S	R
Rifampin	R	S	R	R	R	S	S	S	R	R	R
Penicillin	S	R	R	S	R	S	S	R	S	S	R
Mezlocillin	R	R	R	R	R	S	S	R	R	S	R
Ampicillin	S	R	R	R	R	S	R	I	S	I	R
Cefotaxime	R	S	I	I	R	I	I	I	R	R	S
BacitracinR	S	R	R	R	R	R	S	R	R	S	R
GentamicinS	R	R	R	R	R	R	R	S	R	R	R
KanamycinS	I	R	I	R	I	R	R	S	R	S	R
TreptomycinS	S	R	S	S	R	I	S	S	R	S	S

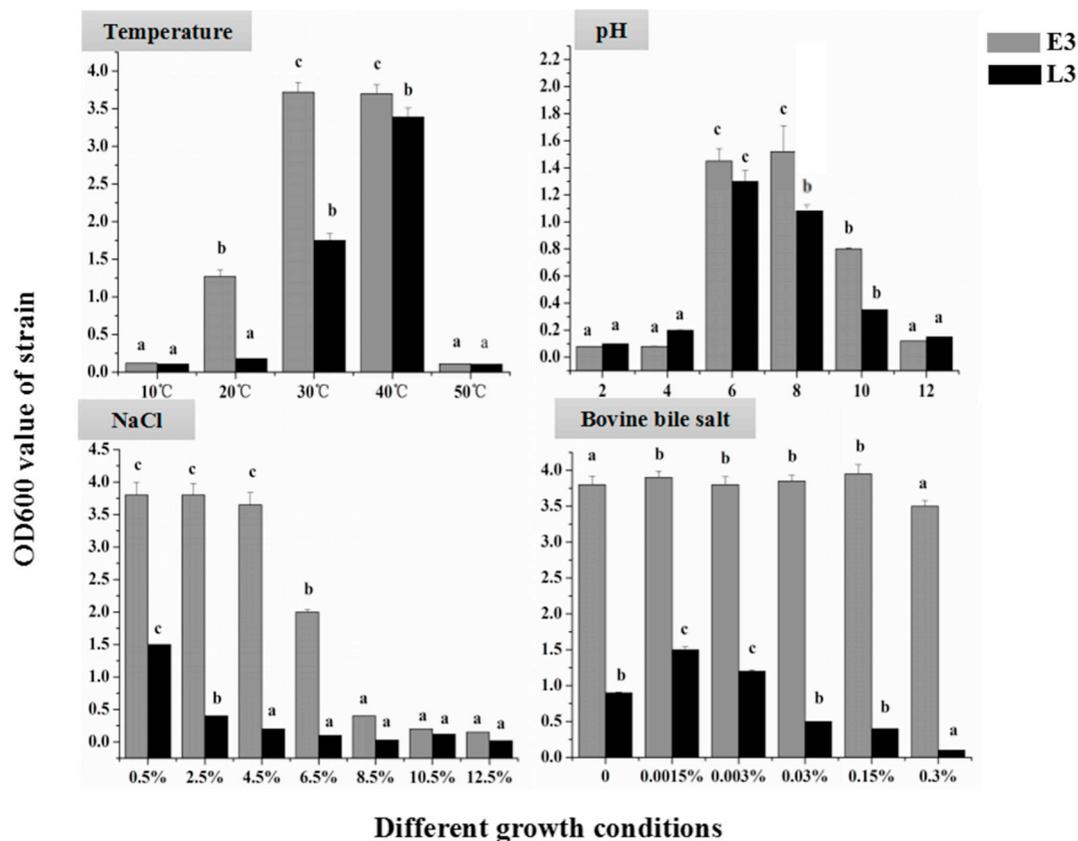


Fig. 2. Biological characteristics of E3 and L3, including temperature, pH, NaCl concentration, and bovine bile salt tolerance concentration. Values are presented as means ± standard deviation (n = 3), and significant (p < 0.05) differences are indicated by different letters (a, b, and c).

group were better than those in control group. And there were few particles in epithelial cells in control group, the cytoplasm of probiotics group was filled with high density granules and showed active secretory state. Among which, the repair effect of intestinal mucosa in E3 group was better than that in L3 group (Fig. 6).

3.7. Intestinal microbial community of *L. vannamei*

The 1st day after the end of the 28-day feeding with probiotics, the AWCD (average change rate of color per hole) of group E3 and group L3

was significantly higher than the control group, and the AWCD of intestinal microorganism in group E3 was higher than that in group L3. On the 5th day, the AWCD of E3 group and L3 group tended to be consistent, but they were still significantly higher than that of the control group, and on the 10th day, the microorganism AWCD of shrimp in L3 and E3 group and the control group were almost the same (Fig. 7). The results suggested that the total ability of intestinal microbes to utilize carbon sources of probiotic groups were significantly higher than that in the control group till the 10th day. The diversity index table of intestinal microflora showed that, compared with the

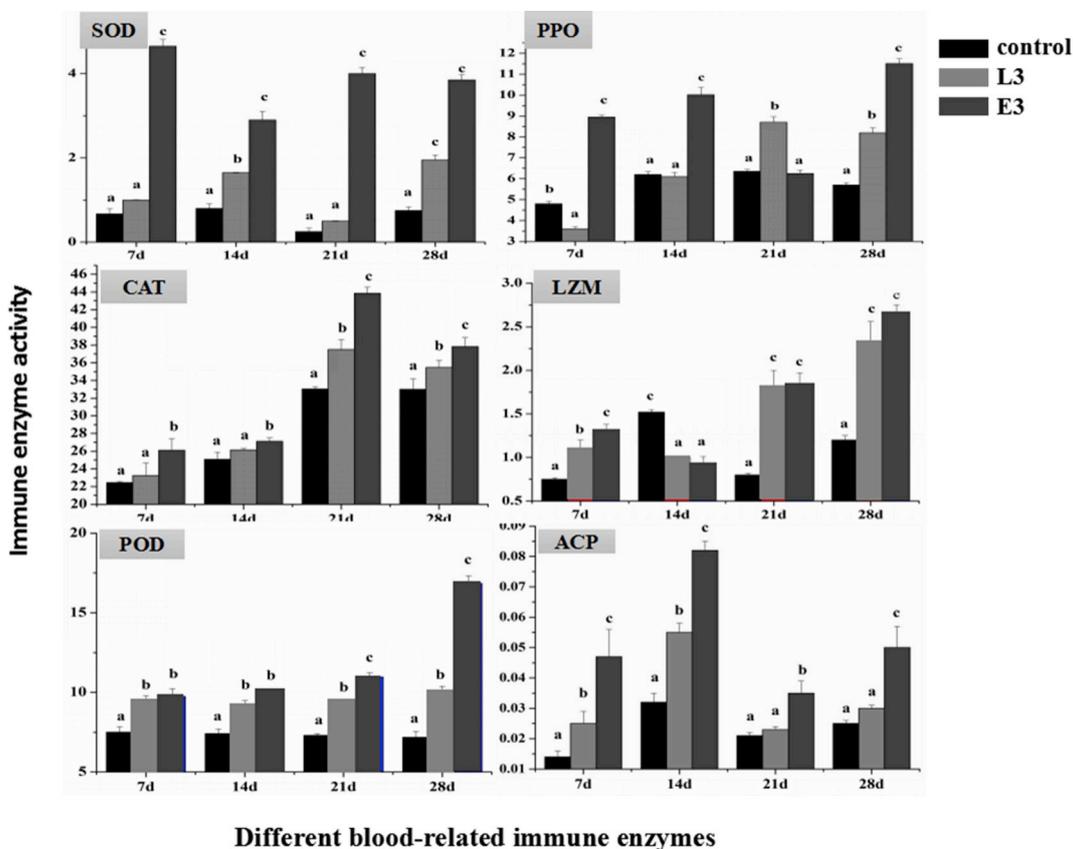


Fig. 3. Activity of blood nonspecific immune-related enzymes in *L. vannamei*, including polyphenol oxidase, superoxide dismutase, acid phosphatase, lysozyme, peroxidase, catalase. Values are presented as means \pm standard deviation ($n = 3$), and significant ($p < 0.05$) differences are indicated by different letters (a, b, and c).

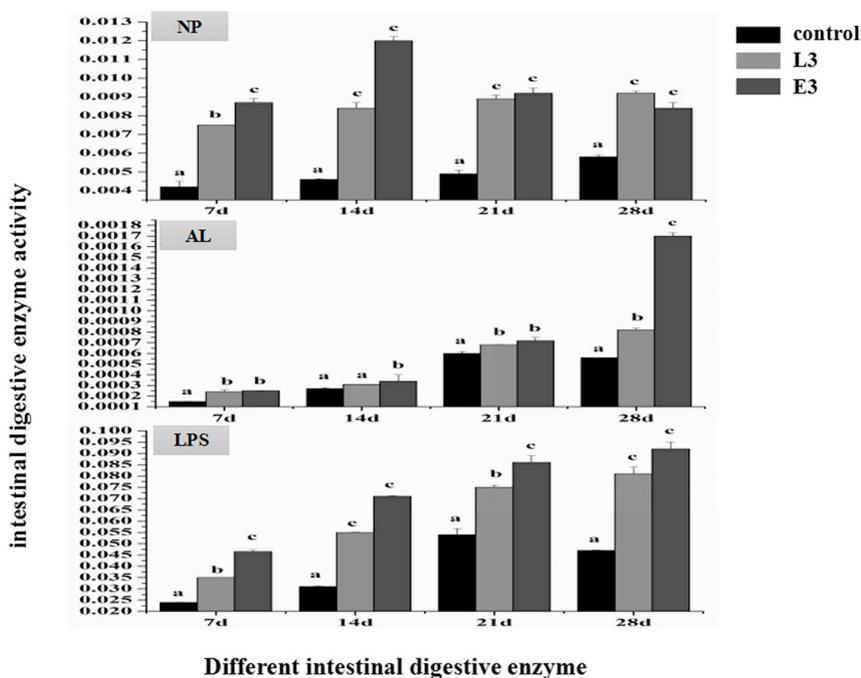


Fig. 4. Activities of digestive enzymes in the intestine of *L. vannamei*, including neutral protease, amylase, lipase. Values are presented as means \pm standard deviation ($n = 3$), and significant ($p < 0.05$) differences are indicated by different letters (a, b, and c).

control group, the Shannon index of the intestinal microorganism community of E3 group was significantly reduced at the 1st and 5th days after the 28-day feeding, the Simpson index and McIntosh index

were significantly increased, and there was no significant difference in the 10th day sampling of the E3 group; The Simpson index and McIntosh index of L3 group was significantly increase compared with

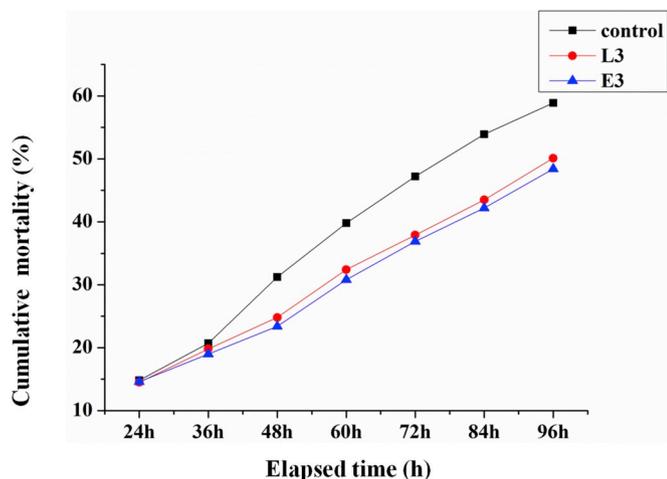


Fig. 5. The cumulative mortality of *L. vannamei* at 24 h, 36 h, 48 h, 60 h, 72 h, 84 h and 96 h after WSSV infection.

the control group at the 1st and 5th days, but there was no significant difference in the 10th day sampling (Table 2). The results indicated that probiotics could only affect the metabolic diversity of intestinal microbial communities of *L. vannamei* in short term, and the microbial communities would tend to be consistent with those of the control group after this period, and the experiment showed that the duration of probiotics in the intestinal of *L. vannamei* was at least 5 days.

3.8. Statistical analysis

Results are presented as means \pm standard deviation from triplicate experiments. The data were analyzed for significance by one-way ANOVA and then followed by Duncan multiple comparison tests using SPSS Software version 11.0. P value less than 0.05 was considered statistically significant.

4. Discussion

Microecological agents can not only enhance the immune function of aquatic animals, reduce the incidence of diseases, but also purify the aquaculture waters, promote the development of aquaculture industry towards a sustainable direction, so it is more and more widely used [19–21]. However, the probiotic species of some raw materials used in the manufacture of microecological agents are different, resulting in the complexity of the products and the lack of pertinence and effectiveness in the probiotics of aquatic animals [22]. Therefore, in this experiment, the intestinal microflora of healthy *L. vannamei* was isolated, and further through the antagonistic experiment, exocrine digestive enzyme activity experiment and the safety experiment to obtain the candidate probiotics. Candidate probiotics with antagonistic and exocrine digestive enzymes were identified to be *Enterobacter hormaechei* (E3) and *Lactobacillus* (L3) respectively, then the biological characteristics of E3 and L3 were studied for further amplification culture and preliminary application in breeding experiment. The indigenous probiotics may be targeted to solve the problems of *L. vannamei* diseases or to promote the growth of the shrimp.

It has been reported that *Enterobacter hormaechei* can cause infection in animals and humans under certain conditions. It is a conditional pathogen and can be isolated from the wound infection of animals and humans. In addition, *Enterobacter* species have been found to be among the top five Gram-negative bacilli that cause hospital infections [23]. Isolates of the *Enterobacter cloacae* complex induce apoptosis of human intestinal epithelial cells [24]. However, the probiotic role of *Enterobacter hormaechei* and its application in the preparation of antimicrobial agents have also been reported in relevant literature, which can prevent and control *Vibrio alginolyticus* or *Vibrio parahaemolyticus* preparations, and can be used as a beneficial microbial preparation to substitute chemical drugs and to promote the development of aquaculture [25,26]. In this experiment, *Enterobacter hormaechei* (E3), which was screened from the intestine of *L. vannamei*, showed no hemolytic activity and pathogenicity, what's more it played a superior role on the growth, immune and digestive enzyme activity and intestinal flora after feeding of *L. vannamei*. Probiotics have many amazing effects, but these

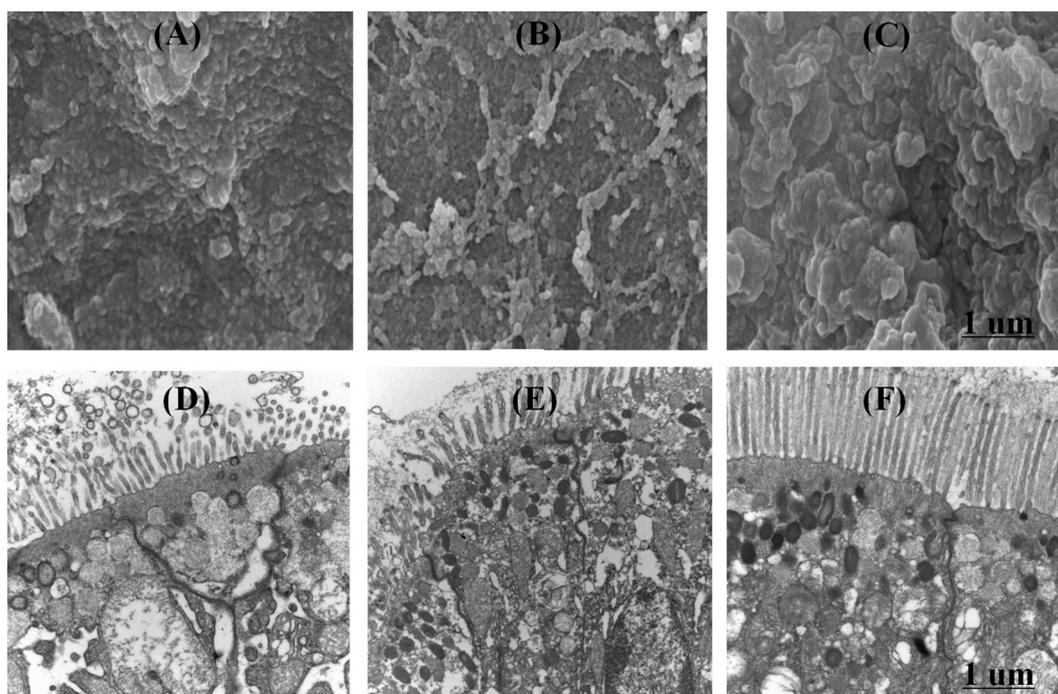
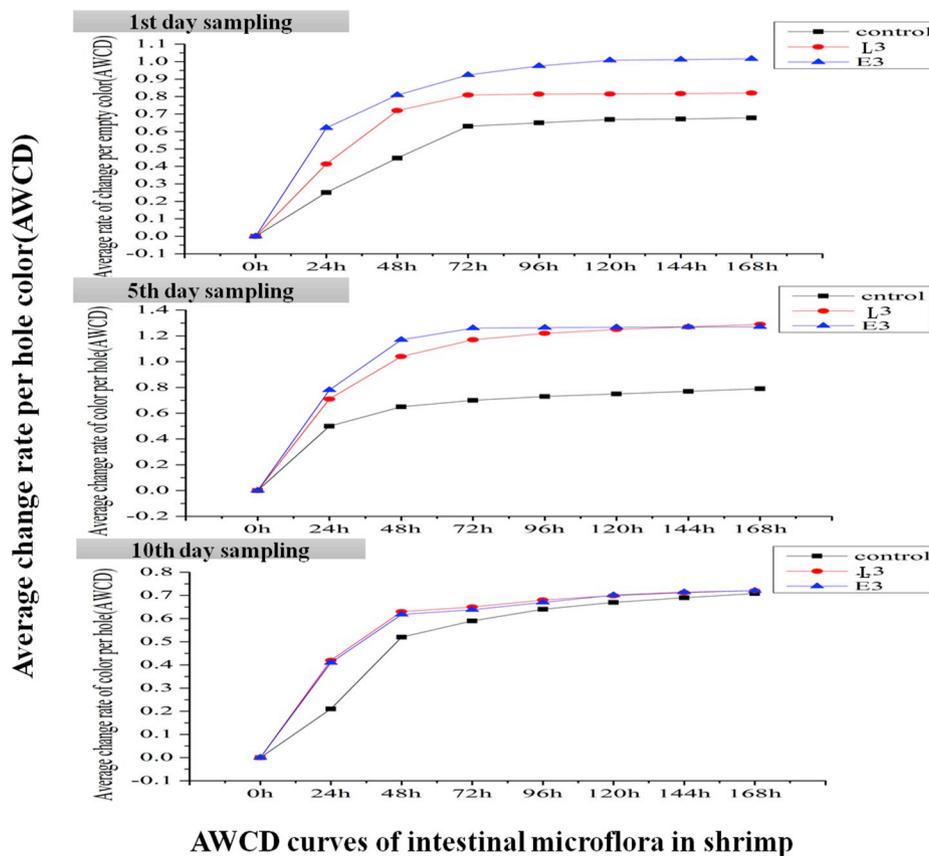


Fig. 6. Electron microscopic results of intestinal sections of *L. vannamei*. (A). Control group (SEM); (B). L3 group (SEM); (C). E3 group (SEM); (D). Control group (TEM); (E). L3 group (TEM); (F). E3 group (TEM).



AWCD curves of intestinal microflora in shrimp

Fig. 7. The changes of AWCD in intestinal microorganism community of *L. vannamei* were continuously detected at 1-st day, 5-th day and 10-th day sampling after the end of the 28-day feeding with probiotics.

Table 2
The microbial community functional diversity index.

Sampling time	experimental group	diversity indices		
		shannon index	simpson index	mcIntosh index
1- st day sampling	E3	3.050 ± 0.033*	56.697 ± 0.876*	6.046 ± 0.331*
	L3	3.413 ± 0.231	62.801 ± 0.221*	5.403 ± 0.562*
	control	3.363 ± 0.017	38.393 ± 0.289	4.012 ± 0.389
5- th day sampling	E3	3.155 ± 0.272 *	82.314 ± 0.223*	7.931 ± 0.291**
	L3	3.394 ± 0.013	79.949 ± 0.086*	8.091 ± 0.023**
	control	3.364 ± 0.103	69.328 ± 0.381	3.949 ± 0.048
10- th day sampling	E3	3.293 ± 0.031	91.556 ± 0.852	6.492 ± 0.212
	L3	3.304 ± 0.129	97.015 ± 0.496	6.474 ± 0.539
	control	3.308 ± 0.059	94.050 ± 0.344	6.324 ± 0.542

Note: * the difference between the experimental pool and the control pool was significant ($p < 0.05$), Values are presented as means ± standard deviation (n = 3).

different effects are highly dependent on strain specificity. A large number of clinical studies have shown that a specific probiotic strain has some definite function but does not mean that different strains of the same probiotics must have the same or similar functions [27]. Our experimental results seemed to confirm the above statement.

The humoral immune defense mechanism of *L.vannamei* maximizes its nonspecific immune response, which mainly depends on non-specific enzymes in hemolymph, immune active factors such as alkaline phosphatase, peroxidase, etc., in which phagocytosis of blood cells mediates agglutination [28–30]. Solidification, bactericidal substances and other reactions in the process of opening the immune defense mechanism, and then promote its own virus killing power, maintain the best state of defense [31–34]. In this study, immune enzyme (SOD, PPO, ACP, POD, CAT, LZM) activity of two experimental groups fed with *Enterobacter hormaechei* (E3) and *Lactobacillus* (L3) probiotics was significantly higher than that of the control group, at the same time, the cumulative

mortality rates in probiotics groups were significantly lower than that in the control group after WSSV infection, which was consistent with the experimental results that probiotics could improve the activity of nonspecific immune related factors and the antiviral ability in *L.vannamei* to some extent.

The growth of aquatic animals depends on the digestion, absorption and transformation of various nutrients in the digestive tract. These complex and diverse processes are carried out using digestive enzymes in the gut [35]. The digestive enzyme activity can be used to measure the digestive level of aquatic animals, and then to observe whether it is consistent with the growth index of aquatic animals. The application of probiotics instead of antibiotics in aquaculture has made more and more scholars study the digestive enzyme activities in the intestine of aquatic animals more deeply and clearly [36–38]. The results of this experiment showed that the protease, amylase and lipase activity in the intestine of E3 and L3 groups were significantly increased with the

passage of breeding. It may be caused by digestive enzyme secretion from E3 and L3 or by the strengthened secretion from cells stimulated by these two probiotics, or by the combinations of the two factors. And the results of intestinal electron microscope showed that the intestinal mucosa layer was tight and the endocrine state was active in probiotics groups and this is consistent with the digestive enzyme results. In this study, the weight gain rate was significantly higher among shrimps fed with diets supplemented with 10^7 CFU g^{-1} E3 and L3. This result suggested that the digestion and absorption of nutrients was more effective in the E3 and L3 group than the control group, which was consistent with the result of the intestinal tract digestive enzyme activity determination and the result of intestinal electron microscope. And conclusion can be made that the intestinal tract of *L.vannamei* is an important organ to digest food and absorb nutrient.

There is a complex microbial system in the intestinal tract of animals. There are many kinds of microbes and complex community structure [39]. The various groups not only depend on each other to live together, but also restrain each other. Diversity is an important indicator used to describe microbial communities. The diversity in this paper is based on the analysis of the diversity of carbon sources in the intestinal tract of *L.vannamei* based on Biolog-ECO method [40–43]. In this study, the average change rate of color per hole of the experiment group supplemented with E3 and L3 was increased significantly compared with the control group, indicated that E3 and L3 enhanced the intestinal microbial activity. The overall ability to use carbon in the intestinal microflora of *L.vannamei* was significantly enhanced, indicated the digestive enzymes secreted by E3 and L3 enhanced the digestion and absorption ability of the shrimp intestinal tract, and thus promoted the rapid growth of shrimp, this result further proved the results of the digestive enzyme activity determination and the shrimp growth measurement previously. There were significant differences in the intestinal microbial diversity index (including Shannon, Simpson, and McIntosh index) after the end of the 28-day feeding with E3 and L3, compared with the control group, till to the 5th day. However, no distinct difference at the 10th day sampling between the experiment groups and the control group, suggested that the effect of probiotics on intestinal microflora of *L.vannamei* was at least 5 days, then the diversity difference gradually decreased and tended to be consistent at the 10th day after feeding with probiotics. The result shows that the supplementation of E3 and L3 probiotics in the feed can enhance the competitiveness of the intestinal flora, change the number and structure of the original flora in the intestinal tract, and promote the complex interaction between the microorganism community in the intestines of the shrimp, and then play an important role in protecting or promoting the normal aspects of the constitution of the shrimp. But the effect of adding probiotics on the intestinal flora is periodic, and when it exceeds this cycle, the composition of the flora will return to its original state. This provides a certain reference basis for the application of probiotics in aquaculture, and the interval between feeding probiotics can not exceed its duration period.

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