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ORIGINAL RESEARCH PAPER

Beneficial effects of *Bacillus licheniformis* on the intestinal microflora and immunity of the white shrimp, *Litopenaeus vannamei*

Ke Li · Tianling Zheng · Yun Tian · Feng Xi · Jianjun Yuan · Guozheng Zhang · Huasheng Hong

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Abstract When *Bacillus licheniformis* was administered to the white shrimp, *Litopenaeus vannamei*, although the total bacterial counts in the intestinal tract of the shrimp remained constant, *Vibrio* numbers significantly decreased ($P < 0.05$). Haemocyte counts together with phenoloxidase and superoxide dismutase activities of the shrimp were significantly higher ($P < 0.05$) in treatments than in the control. Thus, administration of *B. licheniformis* can improve the white shrimp's intestinal microflora and its immune ability.

Keywords Bacillus licheniformis · Immunity · *Litopenaeus vannamei* · Probiotics

K. Li · T. Zheng (✉) · Y. Tian · F. Xi · G. Zhang
MOE Key Laboratory for Cell Biology and Tumor Cell Engineering, School of Life Sciences, Xiamen University, Xiamen 361005, China
e-mail: wshwzh@xmu.edu.cn

K. Li · T. Zheng · F. Xi · H. Hong
State Key Laboratory of Marine Environmental Science, Xiamen University, Xiamen 361005, China

J. Yuan
Department of Biology, Quanzhou Normal College, Quanzhou 362000, China

Introduction

World commercial shrimp production, including white shrimp, has been affected severely by *Vibrio* spp. and viruses during the past two decades (Lo et al. 2003). A number of preventive measures have been used to improve production levels, such as the use of antibiotics, vaccines and probiotics. Unfortunately, antibiotics and vaccines have their own obvious shortcomings, and thus, because of their safety and efficiency, probiotics have become the focus of attention.

Bacteria used as probiotics include *Lactobacillus* spp. and *Bacillus* spp. Among the large number of probiotic products available today, the genus *Bacillus* is widely used for the following advantages: (1) Spores of the genus *Bacillus* are consistently extremely resistant to external physical and chemical insults because of their special structural organization and, in part, this determines their exceptional longevity in the environment (Henriques and Moran 2000; Nicholson et al. 2000); (2) *Bacillus* spp. usually secrete many exoenzymes (Moriarty 1998).

However, recent studies have indicated that most of these products are mislabeled and carry species of *Bacillus*, which may contain toxin producing genes (Duc et al. 2004). Consequently, these results have given rise to concern about the safety of *Bacillus* products. A more rigorous

selection process is thus required for *Bacillus* probiotic candidates.

Based on these concerns, the purpose of this study was to obtain a safe and effective *Bacillus* strain available for shrimp farming, and also to investigate how the shrimp immune system and its intestinal microflora respond to *Bacillus* probiotic stimulation.

Materials and methods

Isolation and identification of probiotic strains

Several surface sediment samples of Xiamen shrimp ponds were diluted 1:10 (w/v) with sterile buffered peptone water and shaken (150 rpm). Aerobic spore-forming isolates were then selected by incubation at 80°C for 20 min, and 0.1 ml samples of four serial dilutions (1/10) in buffered peptone-water were plated in duplicate on Luria-Bertani (LB) plates. More than 150 *Bacillus* isolates were obtained using spore staining and the catalase test. One strain was identified as *Bacillus licheniformis*, based on a series of physiological and biochemical parameters. This identification was confirmed by 16S rRNA gene sequencing.

The *B. licheniformis* selected was tested for its ability to secrete extracellular macromolecule digesting enzymes. Based on this property, this strain was used as a candidate probiotic bacterium.

Shrimp experiments

Litopenaeus vannamei juveniles (5–6 g), obtained from a private shrimp farmer in Xiamen, China, were acclimatized in the laboratory for two weeks in 12 PVC tanks (each 100 l) before experimentation. All the tanks received continuous aeration, and 50% of the water was exchanged daily to maintain quality. During the experiments, water ranged from 25–28°C, pH 7.8–8.2 and salinity was maintained at 0.8–1%. Shrimps were fed at 3% body weight three times daily.

Bacterial inocula

B. licheniformis was cultured on Luria-Bertani (LB) media at 28°C overnight with continuous agitation. The culture was centrifuged (8,000*g*, 10 min) and the cells washed three times in NaH_2PO_4/Na_2HPO_4 buffer (0.1 M, pH 7.0). The cell density was calculated from the OD_{600} value and also correlated to the colony forming unit (c.f.u.) count using serial dilution and spread plate techniques.

The shrimps were challenged with four treatments and there were three replicates per treatment: One treatment (C group) was fed with an unaltered diet (control). In the other three treatments (T1, T2 and T3 groups), the *B. licheniformis* suspension was added to the tanks to give concentrations of 10^3, 10^4 and 10^5 c.f.u. ml^{-1}, respectively. Following the 40 days feeding period, shrimps at the inter-molt stage were randomly sampled for immunological evaluation and examination of the intestinal microflora.

Evaluation of microbiological count

The digestive tracts of shrimp were collected, homogenized and serially diluted with sterilized normal saline solution. Suspensions (0.1 ml) were spread in triplicate on nutrient agar (with 1% w/v NaCl) and thiosulfate/citrate/bile/salt (TCBS) agar to obtain the c.f.u. counts, which were measured after 48 h at 28°C. Nutrient and TCBS agars were used to detect the total bacteria and *Vibrio* sp. respectively.

Immunological techniques

Haemolymph (100 μl) was withdrawn from the ventral sinus of each shrimp into a 1 ml sterile syringe (25 gauge) containing 100 μl pre-cooled (4°C) anti-coagulant solution (trisodium citrate 30 mM, Nacl 0.34 M, EDTA 10 mM, pH 7.55, osmolality adjusted with glucose to 780 mOsm kg^{-1}). Four to six shrimp haemolymphs were centrifuged (2,650*g*, 10 min), and the supernatants were used for analysis of protein concentration, phenoloxidase (PO) activity and superoxide dismutase (SOD) activity. All of the tests described above were conducted in triplicate.

The total haemocyte count (THC) and the differential haemocyte count (DHC) were measured using a haemocytometer and a

phase-contrast microscope (40× magnification) as described by Muñoz et al. (2000).

Plasma protein concentration was evaluated according to the Lowry method with BSA as the standard.

SOD activity was measured using the method of Sun et al. (1988). One unit of SOD is defined as the amount of protein that inhibits the rate of nitroblue tetrazolium (NBT) reduction by 50%. Data are expressed as U ml^{-1}.

PO activity was measured spectrophotometrically by recording the formation of dopachrome, produced from L-dihydroxyphenylalanine (L-DOPA), based on the procedures of Liu and Li (1998). An increment in OD at 490 nm of 0.001 per min under these conditions was defined as one unit of PO activity.

Statistical analysis

The results were expressed as means ± standard deviation, and the data were analysed, using Duncan's new multiple range test for significant differences among treatments, with an SPSS package. A level of $P < 0.05$ was accepted as statistically significant.

Results

Microbiological analysis

At the end of the experiment (40 days), the total bacterial counts in the three probiotic treatments, which ranged from 3.9×10^6 to 5.1×10^6 c.f.u./g digestive tract, were not significantly different from the control (Fig. 1). However, *Vibrio* counts in the digestive tract used in the three experimental groups were significantly lower than those in the control, *Vibrio* counts were lowest (11.6×10^4 c.f.u./g digestive tract) in T3, while the control reached 26×10^4 c.f.u./g digestive tract (Fig. 2).

Immunity parameters

The THC in the haemolymphs of *L. vannamei* fed on different *B. licheniformis* supplemented diets are illustrated in Table 1. The THC was significantly higher in shrimp fed on diets supplemented with *B. licheniformis* than in the control. Hyaline cells (HC), semigranular cells (SGC) and granular cells (GC) content each showed the same trend as

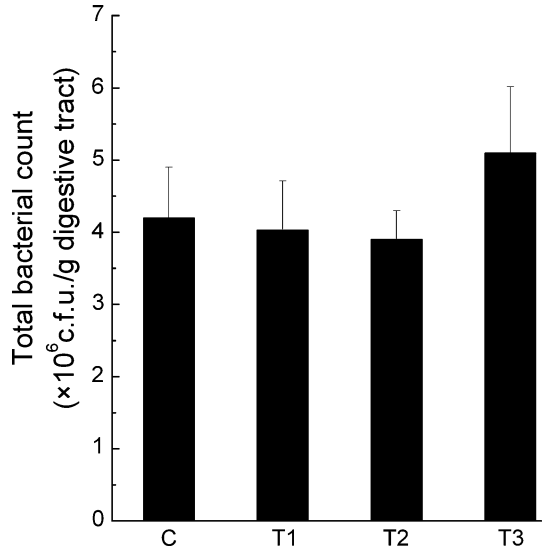

Fig. 1 Total bacterial count in digestive tracts of *L. vannamei* reared with and without *B. licheniformis* added to the water. Mean ± SD indicated. C-controls (no probiotic added); T1-probiotic added to water (10^3 c.f.u. ml^{-1}); T2-probiotic added to the water (10^4 c.f.u. ml^{-1}); T3-probiotic added to the water (10^5 c.f.u. ml^{-1})

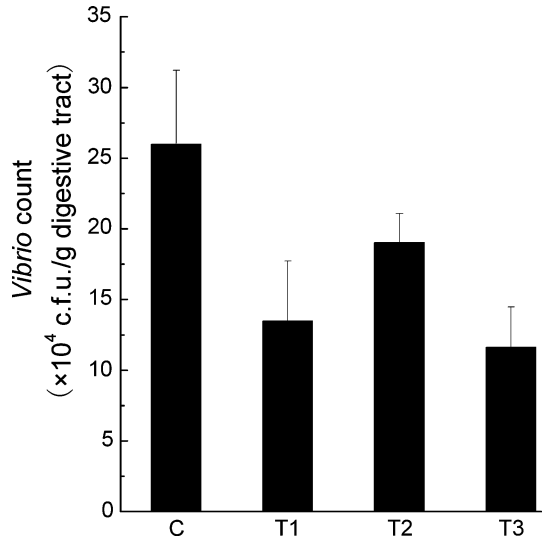

Fig. 2 *Vibrio* count in digestive tracts of *L. vannamei* reared with and without *B. licheniformis* added to the water. Mean ± SD indicated. C-controls (no probiotic added); T1-probiotic added to the water (10^3 c.f.u. ml^{-1}); T2-probiotic added to the water (10^4 c.f.u. ml^{-1}); T3-probiotic added to the water (10^5 c.f.u. ml^{-1})

Table 1 THC (total haemocyte count), HC (hyaline cells), SGC (semi-granular cells) and GC (granular cells) in *L. vannamei* reared with and without *B. licheniformis* added to the water

Haemocyte	C	T1	T2	T3
THC ($\times 10^5$cells ml^{-1})	18.7 ± 4.6	29.2 ± 2.6	45.8 ± 1.0	41.8 ± 3.8
HC ($\times 10^5$cells ml^{-1})	7.2 ± 3.0	10.0 ± 1.5	16.2 ± 4.3	14.5 ± 5.5
SGC ($\times 10^5$cells ml^{-1})	3.8 ± 1.3	9.5 ± 2.0	13.2 ± 0.76	12.5 ± 5.3
GC ($\times 10^5$cells ml^{-1})	7.7 ± 2.8	9.7 ± 2.6	16.5 ± 3.1	14.8 ± 2.1

Mean ± SD indicated. C-controls (no probiotic added); T1-probiotic added to the water (10^3 c.f.u. ml^{-1}); T2-probiotic added to the water (10^4 c.f.u. ml^{-1}); T3-probiotic added to the water (10^5 c.f.u. ml^{-1})

the THC. Statistical differences in semigranular cells were not found among the shrimp of the three groups inoculated with probiotic bacteria. The THC were highest (46×10^5 cell ml^{-1}) in the T2 group, and lowest (19×10^5 cell ml^{-1}) in the control group.

No significant differences in protein concentration were observed between shrimp fed on diets supplemented with probiotic and the control group ($P > 0.05$), though the shrimp fed on all three levels of *B. licheniformis* supplemented diets had slightly higher protein concentrations than shrimp fed on the control diet (Fig. 3). Protein concentration ranged from 91 mg ml^{-1} to 96 mg ml^{-1}.

Figure 4 shows a significant increase of PO activity in the haemolymph supernatant of *L. vannamei* reared with *B. licheniformis* added to the water, compared to the control group ($P < 0.05$). The PO activity of the shrimp progressively increased with the higher concentrations of *B. Licheniformis*, from T1 to T3, and the largest increase was 15.6 U min^{-1} in the T3 group.

SOD activity was lower after the administration of *B. Licheniformis* in the T1 and T2 groups compared to the control group ($P > 0.05$), but significant enhancement was observed in the T3 group ($P < 0.05$) (Fig. 5).

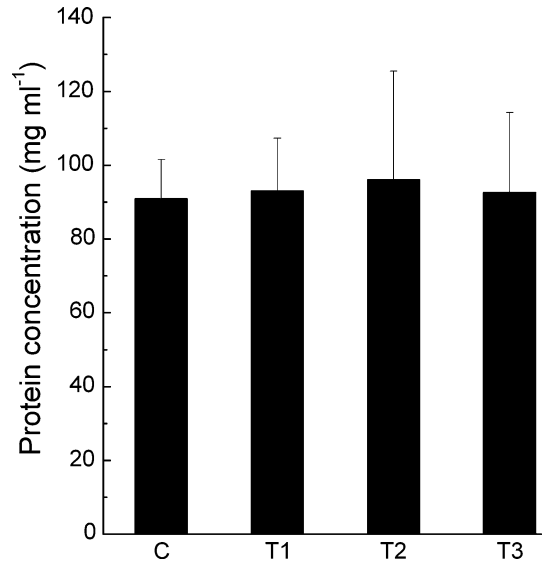

Fig. 3 Protein concentration in haemolymph supernatant of *L. vannamei* reared with and without *B. licheniformis* added to the water. Mean ± SD indicated. C-controls (no probiotic added); T1-probiotic added to the water (10^3 c.f.u. ml^{-1}); T2-probiotic added to the water (10^4 c.f.u. ml^{-1}); T3-probiotic added to the water (10^5 c.f.u. ml^{-1})

Discussion

A survey of microbial populations in the sediment from a shrimp culture region revealed that over 50% (by cells) of the biomass were *Bacillus* (Wang et al. 2004). This indicated that *Bacillus* is important in the microecosystem of the aquaculture environment. In the present work, we isolated and identified a strain of *B. licheniformis* from the sediment of a local shrimp pond, and we proposed a series of assays for this *B. licheniformis* in order to investigate its use as a probiotic. Bacterial counts in the digestive tract and the non-specific immune responses of groups of *L. vannamei* fed for 40 days with three different dosages of this *B. licheniformis* were evaluated.

This study showed that the number of *Vibrio* spp. in the intestine of shrimp decreased significantly with probiotic treatment, while the total bacterial counts remained the same both with and without probiotic treatment. This finding indicated that *B. licheniformis* may have played a role

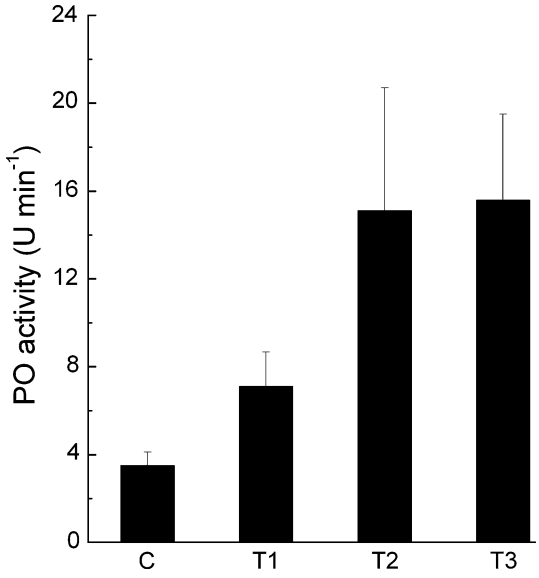

Fig. 4 PO activity in haemolymph supernatant of *L. vannamei* reared with and without *B. licheniformis* added to the water. Mean ± SD indicated. C-controls (no probiotic added); T1-probiotic added to the water (10^3 c.f.u. ml^{-1}); T2-probiotic added to the water (10^4 c.f.u. ml^{-1}); T3-probiotic added to the water (10^5 c.f.u. ml^{-1})

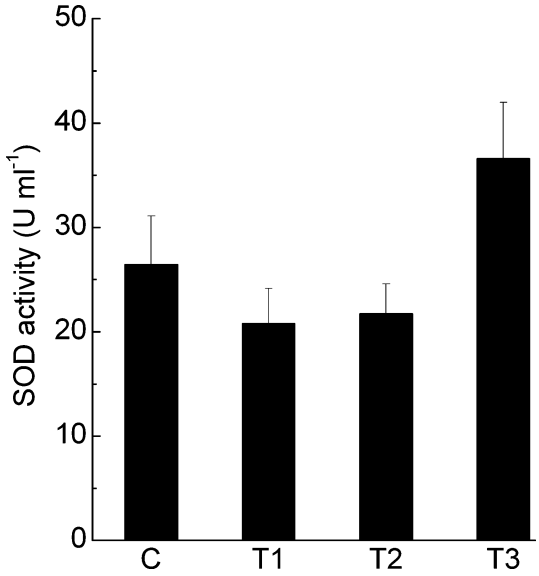

Fig. 5 SOD activity in haemolymph supernatant of *L. vannamei* reared with and without *B. licheniformis* added to the water. Mean ± SD indicated. C-controls (no probiotic added); T1-probiotic added to the water (10^3 c.f.u. ml^{-1}); T2-probiotic added to the water (10^4 c.f.u. ml^{-1}); T3-probiotic added to the water (10^5 c.f.u. ml^{-1})

as a competitive exclusion agent because its administration changed the bacterial population of the digestive tract and led to a decrease in the population of *Vibrio* spp., a group which has been associated with shrimp pathology (Mohney et al. 1994).

To date, studies concerning the enhancement of the immune ability of shrimps arising from the administration of *Bacillus* are scarce. Our study has demonstrated a promising immune response stimulation of *L. vannamei* with probiotic treatment. Shrimps have three types of circulating haemocytes: hyaline cells, semi-granular cells and large granular cells (Sung et al. 1996) which are important for their association with the recognition and removal of foreign material. In the present study, the three types of haemocytes were significantly higher after the probiotic treatment than in the control group. Rengpipat et al. (2000) and Gullian et al. (2004) have reported a similar phenomenon. Haemocytes are associated with the pro-phenoloxidase (proPO) system involved in encapsulation and melanisation and which functions as a non-self recognition system (Smith and Söderhäll 1983; Johansson and Söderhäll 1989). For shrimps receiving *B. licheniformis*, the PO activity was significantly higher than in the control, which corresponds to an increased THC.

A recent study showed that SOD activity related to the immunity of aquatic organisms being related to the defensive ability of the phagocytes and the whole immune response (Mou et al. 1999; Liu and Li 1998). In our study, shrimps in the T3 group had a significantly higher SOD activity and there was no significant difference among the other three groups. The possible reason is that the dose of bacteria is vital to the final result. Furthermore, protein concentration in the three treatments remained equal to the control value, indicating that supplementation by bacteria does not result in a deterioration of shrimp health.

Conclusion

The present study revealed that *B. licheniformis* administered to *L. vannamei* cultures inhibited *Vibrio* sp. by competitive exclusion, and

improved shrimp immunity by increasing their THC, SOD and PO activity. Thus, *B. licheniformis* can be used as an immunostimulant in aquaculture food supplements in order to increase shrimp immunity.

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