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Effect of probiotic on larvae shrimp (*Penaeus vannamei*) based on water quality, survival rate and digestive enzyme activities

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ABSTRACT

The effect of probiotic, *B. coagulans* SC8168, as water additive on larvae shrimp (*Penaeus vannamei*) based on water quality, survival rate and digestive enzyme activities was investigated at ontogenetic stages (Z_3, M_3, PL_{1-2} and PL_{7-8}). Twelve tanks with three replicates for each treatment group and control group were used. The treatments consisted of three SC8168 levels at an initial concentration of 1.0×10^5 cfu ml^{-1} (T-1), 5.0×10^5 cfu ml^{-1} (T-2) and 1.0×10^6 cfu ml^{-1} (T-3) and one control (without any probiotic), and were conducted every day. Addition of the probiotic significantly increased survival rate ($P<0.05$) for all treatments over controls. However, no significant difference was found between T-2 and T-3. At early larval stages (Z_3 and M_3), protease activity in shrimp was not significantly different among probiotic treatments and control. At the subsequent ontogenetic stages (PL_{1-2} and PL_{7-8}), the highest protease activity was observed in T-2 and there was a significant difference ($P<0.05$) between the treatment and the control. Similar results were observed in T-3 at PL_{7-8} stage ($P<0.05$). Amylase activity in T-2 at Z_3, M_3, PL_{1-2} and PL_{7-8} stages was significantly higher ($P<0.05$) than that in the control. The amylase activity was also increased significantly ($P<0.05$) in T-3 than the control except the M_3 stage. As for the lipase activity, assays showed a significant difference ($P<0.05$) in groups treated with SC8168 as compared with the control except the initial stage (Z_3). However, a concentration response of probiotic strains in T-1, T-2 and T-3 was not observed in the present research. The results from this study suggest that *B. coagulans* SC8168 supplemented at a certain concentration could significantly increase survival rate and some digestive enzyme activities of *P. vannamei* larvae.

1. Introduction

The abuse of antimicrobial drugs, pesticides, and disinfectants in aquaculture has caused the evolution of resistant strains of bacteria and concern of the society (Esiobu et al., 2002; Boyd and Massaaut, 1999). Thus, the use of probiotics in the culture of aquatic organisms is increasing with the demand for more environment-friendly aquaculture practices (Gatesoupe, 1999). Probiotics is the use of microbial supplements to benefit their host (Fuller, 1989). Although application of probiotics in aquaculture seems to be relatively recent (Kozasa, 1986), the interest in such environment friendly treatments is increasing rapidly. Moriarty (1998) proposed to extend the definition of probiotics in aquaculture to microbial "water additives." A growing number of studies have dealt explicitly with probiotics, and it is now possible to survey its state of the art, from the empirical use to the scientific approach (Mohanty et al., 1996; Gatesoupe, 1999; Gomez-Gil et al., 2000; Sharma and Bhukhar, 2000; Wang et al., 2005; Wang, 2007; Wang and Xu, 2006; Vine et al., 2006; Kesarcodi-Watson et al., 2008).

The potential benefits of probiotics in aquaculture ponds include improvement of water quality, enhancement of nutrition of host species through the production of supplemental digestive enzymes, lower incidence of diseases and greater survival, and improved immune response (Boyd and Massaaut, 1999; Verschuere et al., 2000). Although probiotics for human and terrestrial animals are dominantly lactic acid bacteria (LAB), many different genera, including photosynthetic bacteria, yeast, *Bacillus* and Lactobacillus have been evaluated as probiotics in fish and shellfish (Gatesoupe, 1999; Vine et al., 2006; Kesarcodi-Watson et al., 2008). However, few studies have been carried out on spore forming lactic acid producing bacteria such as *Bacillus coagulans* (*B. coagulans*) as probiotics in larvae shrimp.

The principal objective of this study was to obtain information concerning the enzymes present and changes in activities that occur in the digestive tract of the larvae Pacific white shrimp, *Penaeus vannamei*, in response to probiotic, *B. coagulans*, supplemented in water. In addition, the water quality and survival rate were also investigated at some ontogenetic stages with and without probiotic. For the digestive enzyme parameter assays, the protease, amylase and lipase activities were used as indicators.

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2. Materials and methods

2.1. Probiotic strain

The microorganism employed was *B. coagulans* SC8168 obtained from the pond sediment of shrimp in Zhejiang Province, China. They were preserved at the laboratory and we routinely checked its purity during this investigation. The probiotic strain, *B. coagulans* SC8168 on normal nutrient agar by spore staining with the spread plate technique was cultured and counted (Marshall and Beers, 1967). Stock cultures of probiotics were stored at −70 °C (Forma 702, Thermo, USA) in powdered skimmed milk suspension with 25% glycerol prior to use (Cabo et al., 1999).

2.2. Experimental design

Four trials were carried out with larvae shrimp in twelve aerated fiberglass tanks with nature seawater (15 l) at a density of 100 per tank. Shrimp were produced at the Oceanic Institute of Zhejiang and were the offspring of specific pathogen free broodstock. All treatments and controls were repeated in triplicate. The treatments consisted of three SC8168 levels at an initial concentration of 1.0×10^5 cfu ml^{-1} (T-1), 5.0×10^5 cfu ml^{-1} (T-2) and 1.0×10^6 cfu ml^{-1} (T-3) and one control (without any probiotic), and were conducted every day. The selected strain was grown in culture medium in a shaking incubator at 30 °C. After incubation, the cells were harvested by centrifugation (2000 *g*), washed three times with PBS (Phosphate Buffered Saline, pH 7.2, Sangon, China), and re-suspended in the same buffer before use. The same amount of solution without any probiotic was applied in the control tanks when probiotics were applied in the treatment tanks.

The tanks were supplied with running water which had been filtered through the special cotton filter (flow rate: 0.6 l min^{-1}), then passed successively through a tungsten heater and degassing column packed with plastic rings. The temperature was maintained at 25±1 °C and the salinity range of water in the tanks was 32–33‰. For water quality control, temperature and salinity were measured daily. A 16 h dark/8 h light photoperiod was maintained during the entire trial. Shrimp nauplii were fed with a mixture of the microalgae *Chaetoceros* and *Tetraselmis*, which were added daily at a rate of 2×10^6 cells ml^{-1}. At mysis stage, shrimp larvae were fed with *Artemia franciscana* nauplii (Aquacultural Feed Co., Zhejiang, China) at a rate of 5–6 nauplii for each shrimp larva, and 4 times per day until mysis stage 3 (M_3) and at a rate of 10–12 nauplii for each shrimp larva and 5–6 times per day from M_3 through 7–8 days after metamorphosis (PL_{7-8}). Furthermore, the mortality rate of each tank was recorded daily. At the end of the experiment, the percent survival was determined.

2.3. Sampling and analytical methods

The values of pH were measured using the Hach kit (DREL 2400, Hach Company, Colorado, USA). Water samples were collected from the tanks at Z_3, M_3, PL_{1-2} and PL_{7-8} stages for chemical analyses. Water sample collection and on-site measurements were carried out in daylight, between 10:00 am and 12:00 pm. The concentrations of NH_4–N and NO_2–N of water in the tanks were measured according to the procedures of Parsons et al. (1984) and Wei (2002).

Between 30 and 60 shrimps, depending on the development stage, were collected at Z_3, M_3, PL_{1-2} and PL_{7-8} stages from each treatment for enzyme assay. All samples were collected between 10:00 am and 12:00 pm, 2–4 h after feeding. Shrimp were washed with distilled water and immediately frozen at −70 °C in 1 ml Eppendorf tubes until enzyme assays were done. Frozen samples were homogenized in 500 µl ice-cold de-ionized water. Homogenates were centrifuged at 16,000 ×*g* for 10 min at 4 °C. Part of the supernatant was diluted in 10 volumes of ice-cold de-ionized water. Homogenates (crude or diluted) were immediately used for enzyme analysis.

The total soluble protein content was measured in diluted homogenates by the Bradford method (Bradford, 1976) using bovine serum albumin as a standard. Protease activity was evaluated according to Lowry et al. (1951) using Folin-phenol reagent and amylase activity was measured according to Jiang (1982) and Worthington (1993) using iodine solution to reveal non-hydrolyzed starch. Lipase activity was determined based on the measurement of fatty acid release due to enzymatic hydrolysis of triglicerides in stabilized emulsion of olive oil (Borlongan, 1990; Jin, 1995). Enzyme activities were measured as the change in absorbance using a Shimadzu 160-UV spectrophotometer and expressed as specific activity (U mg^{-1} protein).

Analysis of variance (ANOVA) was used to determine the significant ($P<0.05$) difference between the tested groups. All statistics were performed using SPSS for Windows version 11.5 (SPSS, Chicago, USA).

Table 1
The pH values of water treated with (T-1, T-2 and T-3) or without (control) probiotic, *B. coagulans* SC8168, as water additives in shrimp (*Penaeus vannamei*) tanks

Group/treatment	Control	T-1	T-2	T-3
Z_3	7.57±0.12 a	7.50±0.10 a	7.67±0.15 a	7.63±0.15 a
M_3	7.67±0.12 a	7.57±0.06 a	7.70±0.10 a	7.63±0.06 a
PL_{1-2}	7.70±0.10 a	7.63±0.15 a	7.77±0.15 a	7.87±0.15 a
PL_{7-8}	7.63±0.15 a	7.53±0.15 a	7.53±0.06 a	7.73±0.15 a

Z_3: zoea stage 3; M_3: mysis stage 3; PL_{1-2}: postlarvae 1–2 days after metamorphosis; PL_{7-8}: postlarvae 7–8 days after metamorphosis.
Results were presented as means±S.E. of triplicate observations.
Means in the same row with different letters at the same stage were significantly different ($P<0.05$).

Table 2
The NH_4–N concentrations (mg l^{-1}) of water treated with (T-1, T-2 and T-3) or without (control) probiotic, *B. coagulans* SC8168, as water additives in shrimp (*Penaeus vannamei*) tanks

Groups	Control	T-1	T-2	T-3
Z_3	0.0237±0.0035 a	0.0210±0.0020 a	0.0203±0.0025 a	0.0190±0.0026 a
M_3	0.0310±0.0030 a	0.0287±0.0021 a	0.0263±0.0023 a	0.0287±0.0032 a
PL_{1-2}	0.0307±0.0031 a	0.0300±0.0026 a	0.0267±0.0025 a	0.0287±0.0050 a
PL_{7-8}	0.0323±0.0040 a	0.0303±0.0031 a	0.0263±0.0031 a	0.0273±0.0035 a

Z_3: zoea stage 3; M_3: mysis stage 3; PL_{1-2}: postlarvae 1–2 days after metamorphosis; PL_{7-8}: postlarvae 7–8 days after metamorphosis.
Results were presented as means±S.E. of triplicate observations.
Means in the same row with different letters at the same stage were significantly different ($P<0.05$).

3. Results

3.1. Water quality

The effect of probiotic, *B. coagulans* SC8168, on water pH in larvae shrimp tanks was shown in Table 1. The pH value in treated tanks ranged from 7.50±0.10 to 7.87±0.15, while that of the control ponds ranged from 7.57±0.12 to 7.70±0.10. However, there was no significant difference among groups ($P>0.05$) at the same stage. The NH_4–N

Table 3
The NO_2–N concentrations (mg l^{-1}) of water treated with (T-1, T-2 and T-3) or without (control) probiotic, *B. coagulans* SC8168, as water additives in shrimp (*Penaeus vannamei*) tanks

Groups	Control	T-1	T-2	T-3
Z_3	0.0077±0.0015 a	0.0083±0.0015 a	0.0077±0.0015 a	0.0073±0.0006 a
M_3	0.0103±0.0021 a	0.0100±0.0010 a	0.0090±0.0010 a	0.0090±0.0010 a
PL_{1-2}	0.0110±0.0020 a	0.0083±0.0015 a	0.0093±0.0015 a	0.0080±0.0010 a
PL_{7-8}	0.0083±0.0015 a	0.0073±0.0015 a	0.0077±0.0006 a	0.0067±0.0015 a

Z_3: zoea stage 3; M_3: mysis stage 3; PL_{1-2}: postlarvae 1–2 days after metamorphosis; PL_{7-8}: postlarvae 7–8 days after metamorphosis.
Results were presented as means±S.E. of triplicate observations.
Means in the same row with different letters at the same stage were significantly different ($P<0.05$).

concentrations of water treated with (T-1, T-2 and T-3) or without (control) probiotic in shrimp tanks were presented in Table 2. No significant differences were observed ($P>0.05$) throughout the experimental period at the same stage although the higher concentration was determined in the control compared with T-1, T-2 and T-3. Furthermore, there was no significant difference among the three treatments. As for the NO_2–N concentrations, assays showed no difference ($P>0.05$) in groups treated with *B. coagulans* SC8168, as compared with the control although NO_2–N concentration in T-3 had a relatively lower content (Table 3).

3.2. Survival rate

Application of the probiotic, *B. coagulans* SC8168, significantly increased survival rate ($P<0.05$) in all treatments (generally by 7.00–13.10%) over the controls (Fig. 1). A significant decrease in survival rate was found in T-1 (81.67 ± 2.08%) compared with T-2 (86.33 ± 1.53%) and T-3 (85.67 ± 1.15%). However, no significant difference ($P>0.05$) was found between T-2 and T-3.

3.3. Enzyme activities

Specific enzyme activities for protease across all treatments were presented in Fig. 2. At early larval stages (Z_3 and M_3), specific protease activity in shrimp was not significantly different between probiotic treatment and control. In contrast, at the subsequent ontogenetic stages (PL_{1-2} and PL_{7-8}), the highest protease activity (0.21 ± 0.02 U mg^{-1} protein and 0.26 ± 0.02 U mg^{-1} protein, respectively) was observed in T-2 and there was a significant difference ($P<0.05$), as compared with the control (0.16 ± 0.02 U mg^{-1} protein and 0.17 ± 0.01 U mg^{-1} protein, respectively). Similar results were also observed in T-3 at the PL_{7-8} stage ($P<0.05$). However, there was no signifip

As for the lipase activity of shrimp larvae, assays showed significant difference ($P<0.05$) in groups treated with *B. coagulans* SC8168 over the control except the initial stage (Z_3) (Fig. 4). However, a concentration response of probiotic strains in T-1, T-2 and T-3 was not observed in the present research. At Z_3 stage, there was no significant difference ($P>0.05$) in lipase activity between the control and T-3. In contrast, in T-1 and T-2, lipase activities were significantly higher ($P<0.05$) than in the control.

4. Discussion

It is important to provide shrimp with a healthy environment and probiotics has a great deal of potential (Gomez-Gil et al., 2000). Wang et al. (2005) investigated the effect of commercial probiotics on water quality in shrimp, *P. vannamei*, ponds and the results showed that probiotics could significantly reduce the concentrations of nitrogen and phosphorus in pond water compared with the control. In this study, the use of *B. coagulans* SC8168 in shrimp larvae as water additive had shown inconsistent results. In contrast, there was no obvious effect of probiotic on the water quality during the study. This result may be explained by the good water quality and laboratory conditions in this study in contrast to theirs. In fact, the pH values (7.50–7.87) and concentrations of ammonium (0.0190–0.0323 mg l^{-1}) and nitrite (0.0067–0.0110 mg l^{-1}) determined in this study were stable and within acceptable ranges (Boyd and Tucker, 1998).

It was clear from this study that the application of probiotic, *B. coagulans* SC8168 via the water had beneficial effects on the survival rate of shrimp (*P. vannamei*) larvae. The previous study showed that supplementation of the commercial *Bacillus* probiotic significantly increased the survival rate of India white shrimp (*Fenneropenaeus indicus*) in the treatments over the controls (Ziaei-Nejad et al., 2006). A similar finding was obtained by Nogami and Maeda (1992) and Maeda (1992), who isolated a bacterial strain PM-4 (*Thalassobacter utilis*, Nogami et al., 1997) from seawater and inoculated it into blue crab (*P. trituberculatus*) larval rearing tanks at the concentrations of 10^6 cells ml^{-1} and observed a survival of 27.2% in the test tanks compared with 6.8% in the control tank, with no bacteria inoculated. In *P. monodon*, *Bacillus*, used as4(v)1910.6(0)1s42.6(i3(i)14.b3.7(n)4)-2494 ushrvs4(vb2(lts.)4(d.-270.405)15.5(o)18.9.406)0(o)1(s4(v.2(n)1.)4(d.-216.5)15iz)1(s4(v96.98675.4(s

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