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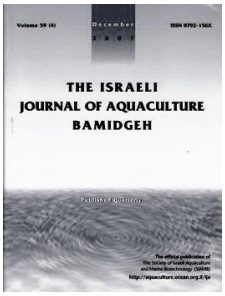

Improved Resistance to *Vibrio parahaemolyticus* in Black Tiger Shrimp *Penaeus monodon* Treated with *Streptococcus phocae* PI80 and *Bacillus subtilis*

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Key words: *Penaeus monodon*, probiotics, immune response, *Streptococcus phocae*, *Vibrio parahaemolyticus*, commercial probiotics

Abstract

The aim of the study was to establish the immune response induced by *Streptococcus phocae* PI80, *Bacillus subtilis*, and commercial probiotics (Uni-Ecosense, EcoForce, Uni-Hatch) in the shrimp, *Penaeus monodon*. Total hemocyte count, phenoloxidase activity, nitroblue tetrazolium (NBT) reductase assay, phagocytic activity, and disease resistance was evaluated for 30 days. Total hemocyte count, phenoloxidase activity, NBT reductase assay, and phagocytic activity significantly increased in juveniles and adults treated with *S. phocae* PI80, a mixture of *S. phocae* PI80 and *B. subtilis*, or a commercial probiotic, but there were no significant differences in live weight of shrimps treated with probiotics or the control. Treatment with *S. phocae* PI80 plus *B. subtilis* enhanced survival of shrimps challenged with *Vibrio parahaemolyticus*. Our study demonstrates that administration of *S. phocae* PI80+*B. subtilis* at 4.6 x 10^8 CFU/ml water induced immune modulation, enhanced immune ability, and increased resistance to *V. parahaemolyticus* in juvenile and adult black tiger shrimp.

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Introduction

Penaeid shrimp culture, especially of black tiger shrimp (*Penaeus monodon*), is economically important worldwide. However the shrimp farming industry faces problems and suffers losses due to diseases caused by pathogenic virus and bacteria (Flegel, 2009). Among the bacterial pathogens, *Vibrio* spp. are dominant in farm-reared shrimp (Lavilo-Pitago et al., 1998). Commercial shrimp culture is hampered by endemic and epidemic infections such as vibriosis and viral diseases (Pattukumar et al., 2010).

Extensive use of antibiotics in shrimp farms and hatcheries for disease control is an ecological threat to coastal areas that are heavily exploited for large-scale cultivation of fish and prawns. Farmers use large quantities of antibiotics as prophylactics, leading to increased propagation of vibrio and, presumably, other bacteria (Moriarty, 1999). Hence, finding alternatives is of premium importance to prevent severe ecological damage.

Microbial intervention through eco-friendly approaches is an alternative method of health management. *Streptococcus* is associated with the mucosa of the gastrointestinal tract of salmonids (Trust and Sparrow, 1974) and lactic acid bacteria are used as probiotics (Ringo and Gatesoupe, 1998; Verschuere et al., 2000; Ganguly et al., 2010). Isolation of a bacterial probiont, *Streptococcus phocae* PI80, from healthy Indian white shrimp, *Fenneropenaeus indicus*, not only improved growth in *P. monodon* postlarvae but also reduced mortality when challenged with luminescent *Vibrio harveyi* and *V. parahaemolyticus* (Swain et al., 2009).

Many microbes with probiotic potential and immune stimulating properties have been studied (Rengpipat et al., 1998, 2000; Gullian et al., 2004; Chiu et al., 2007; Ganguly et al., 2010). The use of probiotic bacteria and immunostimulants, based on the principle of competitive exclusion, is promising (Fuller, 1992). *Streptococcus*-like bacteria are associated with epithelial mucosa in the stomach and small intestine of Arctic charr fed linoleic and linolenic acids (Ringo et al., 1997). In rainbow trout (*Oncorhynchus mykiss*), probiotic bacteria enhance immune activity by improving the barrier properties of mucosa, and by sampling and modulating the production of cytokines, especially in head kidney leukocyte phagocytosis and serum complements (Panigrahi et al., 2005).

Intensive research in the EU, Japan, and USA focuses on commercial probiotic bacterial strains and their health benefits. Rapid growth and high survival during the processing of such strains are required for industrial production. Further, the safety of these strains is very important. While there is no perfect way to quantify the safety of microbial cultures, measurable factors include mucus non-degradation, toxigenicity, infectiousness, activity, or inactive genetic material transfer. Live bacterial cells, enzyme preparations, and yeast extracts are used in aquaculture. However, commercial microbial products may have detrimental effects in field conditions compared to laboratory conditions (Nimrat and Vuthipuhandchai, 2007a), or inadequate efficiency due to an inadequate number of microbes and species in the product. The quantity of probiotic *Bacillus*, *Staphylococcus*, *Streptococcus*, *Micrococcus*, or *Corynebacterium* in a commercial product may be lower than printed on its label (Nimrat and Vuthiphandchai, 2007ab; Srinivas, 2009; Pattukumar et al., 2010). The aim of our current research was to evaluate the potential of *S. phocae* PI80, with and without *B. subtilis*, and selected commercial probiotic strains on growth, survival, and immune response of juvenile and adult *P. monodon*.

Materials and Methods

Bacteria culture. Streptococcus phocae PI80 was previously isolated from the gut of marine shrimp, *Penaeus indicus* (Kanmani et al., 2010). The bacteria were cultured in a sterilized 1-l flask with de Man Rogosa Sharpe (MRS) broth (Himedia, Mumbai, India) for 24 h at 37°C, and then centrifuged at 5000 × *g* for 20 min at 4°C. The pellet was collected and mixed with 0.85% sterile saline and bacteria cells were stored at 4°C until use. The viability of the bacteria mixture was determined by plate counting on MRS agar.

Vibrio parahaemolyticus. A known pathogenic strain *V. parahaemolyticus* (MTCC-451) was obtained from the Microbial Type Culture Collection in Chandigarh, India, for use as a stock bacteria broth. Stocks were cultured on tryptic soy agar (TSA) supplemented with

2.0% NaCl (w/v) for 24 h at 26°C and then transferred to 100 ml tryptic soy broth for 24 h at 26°C. The broth culture was centrifuged at 6000 × *g* for 15 min at 4°C and the bacteria pellets were resuspended in saline solution as a stock solution for the susceptibility study. The pathogenicity of *V. parahaemolyticus* was confirmed by testing on postlarvae and juvenile shrimp.

Commercial probiotics. Four commercial probiotic products were compared with different doses of *S. phocae* PI80: (a) Uni-Ecosense which contains a combination of *Bacillus* strains (*B. polymyxa, B. subtilis, B. licheniformis, B. megaterium, Saccharomyces boulardii*); (b) the starch-based Uni-Hatch, a mixture of *B. subtilis, B. polymyxa*, *B. megaterium,* and *B. licheniformis*; (c) a commercial gray-white powdered *B. subtilis* (batch no. BBS-5-5G02) suspended in deionized water and isolated to form individual colonies by the spread plate technique in nutrient broth; and (d) Eco-Force, a blend of beneficial bacteria containing *Streptococcus faecalis, S. faecium, B. mesentericus, B. natto, B. subtilis, Clostridium butyricum,* and *Saccharomyces cerevisiae*. The first three products were provided as a gift by Uni-Sankyo Ltd., Hyderabad, India, and the fourth as a gift by TIL Biosciences, Division of Tablets (India) Ltd., Chennai, India. All four probiotics were stored at 4°C and viability was checked frequently by the above method.

Experimental design. Two experiments were separately conducted with different weight groups of shrimp: the first with 6-8 g juveniles (7.47±0.351 g) and the second with 25-28 g adults (26.54±1.149 g). Shrimps were obtained from a commercial shrimp farm near Pondicherry, transported to the laboratory, placed in 600-l fiber reinforced plastic (FRP) tanks, and treated with 100 ppm formalin for 15 min. In both experiments, only shrimps in the intermolt stage were used. The molt stage was identified by examining the uropod in which partial retraction of the epidermis could be distinguished. Shrimp were checked for white spot syndrome virus (WSSV) and monodon baculo virus (MBV) using a diagnostic kit (Bangalore Geni, Bangalore, Lot MB3075). Twenty shrimp were randomly distributed into each of 24 FRP tanks containing 400 l filtered seawater, i.e., three replicates of each of eight treatments: (a) unsupplemented control, (b) Uni-Ecosense (5.1×10^8 CFU/g), (c) EcoForce (6.1×10^8 CFU/g), (d) Uni-Hatch (3.5×10^9 CFU/g), (e) *S. phocae* PI80 (10 ml 5.5×10^8 cells/ml) plus *B. subtilis* (10 ml 4.6×10^8 cells/ml), (f) *S. phocae* PI80 (10 ml), (g) *S. phocae* P180 (20 ml), and (h) *S. phocae* PI80 (30 ml). After acclimatization, the bacteria were inoculated into the experimental tanks every three days. The shrimp were fed a commercial pelletized feed (CP Aquaculture, India) containing 41% crude protein, 6% fat, 2% fiber, 13% ash, and 11% moisture three times daily at 10% of their body weight per day. Aeration was provided continuously.

Sampling and analysis. Growth, survival, and water quality were monitored periodically. All shrimp from each tank were weighed at the beginning and end of the 30-day experiment At the same time, survival (%) was determined by counting the individuals in each aquarium, according to the formula 100(final no. shrimp/initial no. shrimp).

Vibrio challenge test. On day 30, surviving juveniles and adults were divided into two groups. One group was challenged with *V. parahaemolyticus* by immersion, the other was an unchallenged control. Challenged juveniles and adults were subjected to a single static exposure to the pathogenic bacterium, *V. parahaemolyticus*. The bacterium was inoculated into 1000 ml TSA with 2% NaCl (w/v) and 0.25% glucose, incubated under shaking conditions for 24 h, and centrifuged at 7000 × *g* for 20 min at 4°C. The supernatant fluid was removed and the bacteria pellet was resuspended in a sterile saline solution (0.85% NaCl) at 10^{-7} CFU/ml. Shrimps were challenged by exposure to *V. parahaemolyticus* in the aquarium water at 10^{-7} CFU/ml. Water was not exchanged thereafter for the duration of the trial. During the challenge tests, water quality, shrimp survival, and number of dead shrimp were recorded every 6 h.

Hemolymph collection. Hemolymph was withdrawn from the ventral sinus of the cephalothorax using a 26-gauge needle fitted to a tuberculin syringe containing 100 µl

ice-cooled hemolymph anticoagulant solution (HAS; 30 mM trisodium citrate, 388 mM sodium chloride, 115 mM glucose, 10 mM ethylenediamine tetra-acetic acid).

Total hemocyte count. Hemolymph (0.1-0.15 ml) was collected from randomly selected shrimp using 200 µl HAS. The hemolymph was gently mixed, hemocytes were counted, and cells/ml were calculated using a hemocytometer (Neubauer chamber) and Nikon Optiphot phase-contrast microscope (400a magnification).

Yeast cell suspension. Commercial grade baker's yeast (*Saccharomyces cerevisiae*) was used to assay phagocytosis. Five hundred mg baker's yeast was suspended in 25 ml PBS (pH 7.0). The suspension was heat-inactivated by autoclaving at 120°C (15 psi). After two washes in PBS by centrifugation, the yeast cells were resuspended in HAS as a 1% packed suspension (Fryer and Bayne, 1989).

Phagocytosis assay and superoxide anion detection. One hundred µl hemocyte suspension was pipetted onto a glass slide and allowed to stand for 30 min to ensure adherence of the cells to the slides. The slides with monolayers of hemocytes were gently rinsed with HAS to remove unattached phagocytes. Fifty µl of opsonised yeast cell suspension was laid on each phagocyte monolayer. After gentle mixing with a pipette, the slides were incubated at room temperature in a humidity chamber for 90 min. Non-phagocytosed yeast cells were removed by washing each slide twice with 100 µl HAS using a pipette. To facilitate quantification of phagocytosed yeast cells, 100 µl 0.5% trypsin solution was dripped onto each slide for 1 min to loosen the adsorbed, but non-phagocytosed yeast cells before rinsing in HAS. This step was introduced to improve the accuracy of the count by ensuring that only phagocytosed yeast cells were counted after evaluating 200 hemocytes in a random microscopic field of each monolayer. Percent phagocytosis = 100(no. phagocytic hemocytes/total no. hemocytes).

Phenoloxidase activity. Phenoloxidase activity in hemolymph samples was determined using L-dihydroxyphenylanine (L-DOPA) as a substrate (Söderhäll and Smith, 1983). Enzyme activity was determined by measuring the absorbance of dopachrome at 490 nm against a blank. Enzyme activity was expressed as units, defined as the amount of enzyme resulting in an increase of 1 mg protein/min at 490 nm.

Intracellular superoxide anion. Nitroblue tetrazolium (NBT) assay is commonly used to measure oxidative burst. The superoxide anion was quantified following Song and Hsieh (1994). One hundred µl hemolymph was collected from all treated and control shrimp and centrifuged at 5000 × *g* for 5 min. The supernatant was discarded and the hemocytes were washed three times with Hank's Balanced Salt Solution (HBSS) followed by staining with an NBT solution (0.3%, 100 µl) for 30 min at 37ºC. The staining reaction was terminated by removing the NBT solution and adding absolute methanol. After three washings with 70% methanol, the hemocytes were air dried and 120 µl 2M KOH plus 140 µl DMSO were added to dissolve the cytoplasmic formazan. Optical density of the dissolved formazan was read at 630 nm and effects of different treatments on the generation of O_2 were compared.

Statistical analysis. Data were analyzed by one-way ANOVA using SPSS vers 16.0. Tukey's multiple range test was used to identify significant differences among the experiments with $p<0.05$ as the confidence level.

Results

Survival. The different treatments had no significant effect on live weight but survival was significantly increased by the administration of probiotics, especially the mixture of *S. phocae* PI80 and *B. subtilis* (Table 1).

Challenge studies. All probiotic-treated shrimp survived the initial challenge. Cumulative mortality of juveniles and adults treated with *S. phocae* PI80 was significantly lower than that of untreated control animals (Fig. 1). Cumulative mortality of shrimps treated with the commercial probiotics was lower than that of the control but higher than that of shrimps treated with *S. phocae* PI80. The cumulative mortality of treated juveniles ranged 16-35%, compared to control juveniles, 66%. Similarly, the cumulative mortality of treated adult shrimps ranged 15-33% while that of the untreated control was 70%.

Table 1. Survival (%±SD) of juvenile and adult tiger shrimp (*Penaeus monodon*) fed diets supplemented with commercial probiotics or *Streptococcus phocae* PI80 for 30 days and infected by immersion in pathogenic *Vibrio parahaemolyticus* at 10^6 CFU/ml for 12 h (n = 3).

Treatment	*Juvenile*	*Adult*
Unchallenged control	50.66±2.08[b]	56.3±2.51[b]
Uni-Ecosense	63±1.52[a]	65.3±1.73[a]
EcoForce	66±1.73[a]	69.3±2.51[a]
Uni-Hatch	68.6±1.52[a]	71.3±1.15[a]
S. phocae PI80 (10 ml)+*B. subtilis*	75.33±2.08[a]	82.3±2.51[a]
S. phocae PI80 (10 ml)	60.3±2.08[a]	67.3±3.21[a]
S. phocae PI80 (20 ml)	66.6±2.51[a]	73.6±4.04[a]
S. phocae PI80 (30 ml)	70.3±3.05[a]	81.6±3.00[a]

Means within columns with different superscripts significantly differ (p<0.05).

Immune indices. Administration of *S. phocae* PI80 and commercial probiotics significantly enhanced the total hemocyte count in juveniles and adults (Fig. 2). Similarly, the phenoloxidase activity and phagocytic activity of juveniles and adults receiving probiotics was significantly higher than in the control shrimp. The highest NBT activity was obtained in juveniles treated with Uni-Ecosense and in adults treated with Uni-Hatch. Juveniles in the *S. Phocae* PI80 treatment groups showed dose dependency.

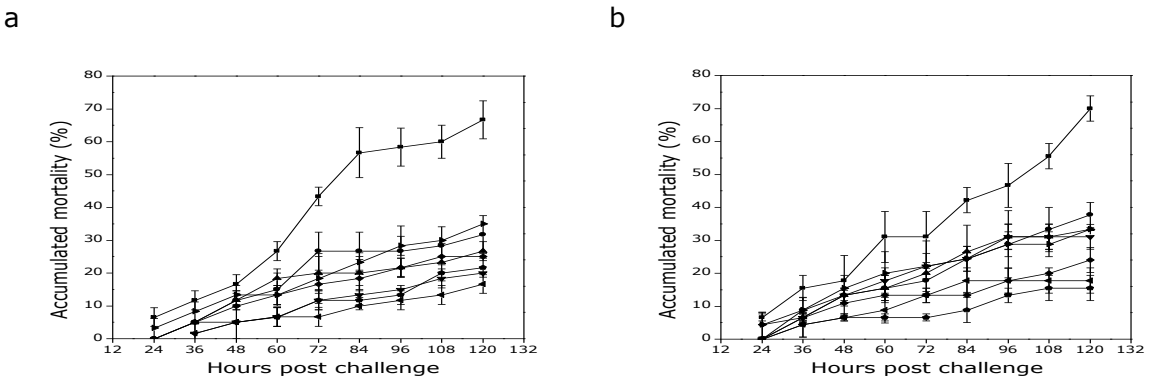

Fig. 1. Accumulated mortality of (a) juvenile and (b) adult *Penaeus monodon* infected with pathogenic *Vibrio parahaemolyticus* at 10^6 CFU/ml for 12 h after being fed diets supplemented with probiotics. All treatments significantly differ from the control. ■ = unsupplemented control, ● = Uni-Ecosense, ▲ = EcoForce, ▼ = Uni-Hatch, ◄ = *Streptococcus phocae* PI80 (10 ml)+*Bacillus subtilis*, ► = *S. phocae* PI80 (10 ml), ◆ = *S. phocae* PI80 (20 ml), ⬟ = *S. phocae* PI80 (30 ml).

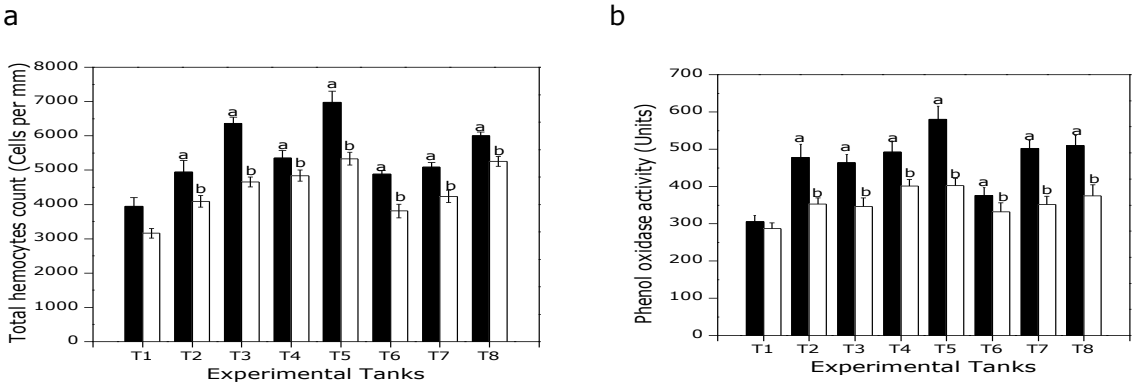

Fig. 2. Effects of probiotics on (a) total hemocyte count, (b) phenoloxidase activity, (c) phagocyte ability in hemocytes, and (d) reduction of nitroblue tetrazolium (NBT) in hemocytes of juvenile and adult *Penaeus monodon* fed diets supplemented with commercial probiotics or *Streptococcus phocae* PI80 for 30 days and infected with pathogenic *Vibrio parahaemolyticus* at 10^6 CFU/ml for 12 h (means±standard deviation, n = 3). Bars with superscripts significantly differ from the control (p<0.05). T1 = unchallenged control, T2 = Uni-Ecosense, T3 = EcoForce, T4 = Uni-Hatch, T5 = *S. phocae* PI80 (10 ml)+*Bacillus subtilis*, T6 = *S. phocae* PI80 (10 ml), T7 = *S. phocae* PI80 (20 ml), T8 = *S. phocae* PI80 (30 ml), ■ = juveniles, □ = adults.

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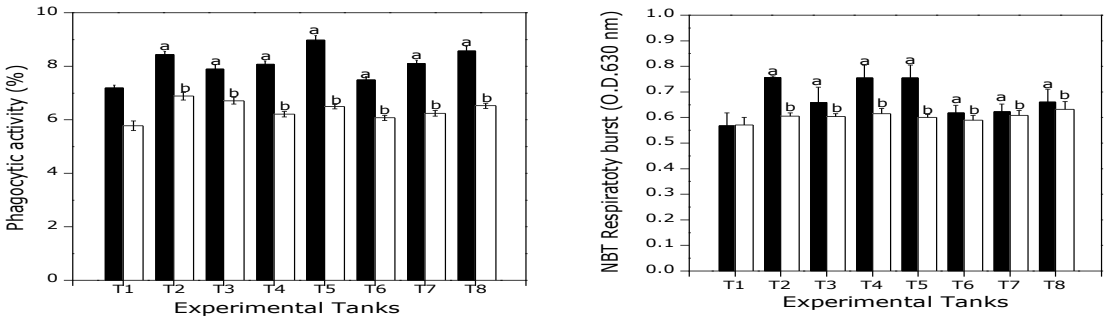

Fig. 2 (cont.).

Discussion

In the present study, we demonstrate that disease resistance and survival of juvenile and adult *P. monodon* in a tank rearing system can be promoted by supplemented probiotics. Administration of *S. phocae* PI80 and a mixture of *S. phocae* PI80+*B. subtilis* significantly enhanced survival. Similarly, survival and growth were higher in *P. monodon* postlarvae tested in two shrimp hatcheries (Swain et al., 2009), confirming that *S. phocae* PI80 is an effective probiotic to control vibriosis in shrimp farms. Many authors have reported significant reductions in mortality of shrimp treated with probiotics. Survival increased in shrimp *Litopenaeus stylirostris* fed the probiotic *Pediococcus acidilactici* and challenged with *Vibrio nigiripulchritudo* (Castex et al., 2010). Administration of *Lactobacillus plantarum* enhanced survival in *Litopenaeus vannamei* challenged with *V. alginolyticus* (Chiu et al., 2007). *Artemia* nauplii enriched with probiotic bacteria and cod liver oil emulsion also resulted in higher survival in *Macrobrachium rosenbergii* (Babitha Rani et al., 2006). Resistance against vibriosis in juvenile *Penaeus vannamei* was increased by supplementing their diet with yeast products (Scholz et al., 1999) and survival significantly improved in *P. monodon* treated with the probiotic bacterium *Bacillus* S11 (Rengpipat et al., 1998, 2000). Cumulative mortality dropped 90% in *P. monodon* postlarvae using *B. subtilis* BT23 (Vaseeharan and Ramasamy, 2003) and survival was high with the probiotic *Bacillus* P62 in *P. vannamei* (Gullian et al., 2004). Similarly, survivability was high in shrimp administered probiotics (Balcázar et al., 2007).

The anti-oxidant defense and immune systems of crustaceans may be closely linked to the response to pathogens (Holmblad and Söderhäll, 1999). Bacterial or viral infection leads to oxidative stress in infected animals within a few hours after infection (Mathew et al., 2007; Sarathi et al., 2007). WSSV infection causes an increase in lipid peroxidation and decrease in anti-oxidant enzyme activities of superoxide dismutase, catalase, glutathione peroxidase, and glutathion-S-transferase in the digestive gland and hemolymph of infected shrimp (Mathew et al., 2007). A similar phenomenon was observed in shrimp *L. stylirostris* infected with *Vibrio nigripulchritudo* (Castex et al., 2010). Decrease of the anti-oxidant defense system leads to accumulation of free radical hydrogen peroxide and hydroxyl radicals, which may cause tissue damage. Infected shrimps recovered and antioxidant defenses returned to initial levels after probiotic treatment (Sarathi et al., 2007; Castex et al., 2010). Similarly, antioxidant activity and total anti-oxidant status increased after treatment with lactic acid bacteria (Kullisaar et al., 2003). In this study, although we did not determine the total anti-oxidant status or anti-oxidant enzyme activity, we used the probiotic culture *S. phocae* whose anti-oxidant activity and reduction of lipid per oxidation were well established in our laboratory (Kanmani et al., 2011; Paari et al., 2011). Thus, the enhancement of survival in challenged shrimp treated with *S. phocae*+*B. subtilis* may be due to enhancement of the anti-oxidant defense system which increased the immune response.

Based on earlier studies of the mechanism of action of probiotics, three hypotheses are proposed: (a) production of anti-bacterial substances and their anti-*in vitro* microbial

activity, (b) competitive exclusion or *in vivo* antagonism, and (c) enhancement of immune responses by increasing total antioxidant status. According to the first hypothesis, the inhibition of *V. parahaemolyticus* in the juvenile and adult shrimp could be attributed to the production of phocaecin, the antagonistic compound that makes pores in the outer membrane of the pathogens leading to the efflux of potassium ions and resulting in their death (Satish kumar and Arul, 2009). A similar mechanism of action was reported by Breukink and Kruijff (2006). The major challenge in the *in situ* demonstration of an antagonistic mode of action of probiotics in aquaculture is to ensure sufficient concentration of the antagonistic compound in the culture supernatant.

In the present study, *in situ* antagonism was achieved by daily application of the probiotics. *S. phocae* PI80 kept the tank water clean and reduced the cannibalism that was prevalent in the control and commercial-probiotic treated groups. In groups treated with commercial probiotics, the water became turbid, requiring frequent exchange of water that might have led to cannibalism, suggesting competitive exclusion or *in vivo* antagonism of the shrimp microorganisms (Guillan et al., 2004; Castex et al., 2008). Probiotic bacteria are generally believed to prevent bacterial infection by competing with pathogenic microbes for adhesion sites and nutrients (Verschuere et al., 2000). *Litopenaeus vannamei* juveniles treated with *Arthrobacter* XE-7 showed resistance when challenged with *V. parahaemolyticus*, indicating competitive exclusion of pathogens established inside the host and stimulation of immune response by the probiotic bacterium (Li, 2008).

Regarding the third hypothesis, mortality was significantly reduced in probiotic-treated shrimp in the present study due to enhancement of the cellular immune responses: phenoloxidase activity, phagocytosis, and superoxide anion activity. There was no significant increase in total hemocyte count of the probiotic-treated shrimp. Similar results were observed by Rengpipat et al. (2000), Gullian et al. (2004), and Chiu et al. (2007). Enhancement of the prophenyl oxidasing system by the probiotics in shrimps is due to stimulation of the prophenyloxidase mRNA level. In *L. vannamei* treated with *Lactobacillus plantarurm*, the prophenyl mRNA level was significantly enhanced and mortality reduced (Chiu et al., 2007). Enhancement of disease resistance may be due to upregulation of prophenoloxidase activity mRNA by shrimp, resulting in increased phenoloxidase activity. The lower hemocyte count may be due to stress caused by food intake, disease outbreak, pollutants, or environment conditions. Despite no difference in total hemocyte count, all other immune parameters remained high in probiotic-treated animals. The mixture of *S. phocae* PI80 and *B. subtilis* significantly enhanced phagocytosis activity, phenoloxidase, and superoxide anion activity in juveniles and adults beyond the administration *S. phocae* PI80 alone. Similarly the commercial probiotics Uni-Ecosense, EcoForce, and Uni-Hatch also enhanced phenoloxidase, superoxide anion detection, and phagocytosis when administered in combination with *B. subtilis, B. polymyxa, B. megaterium,* or *B. licheniformis*: the genus *Bacillus* has peptidoglucan in its cell wall which may increase the immune system of shrimp (Boonyaratpalin et al., 1995).

Administration of *Bacillus* improves the digestive activity synthesis of vitamins, cofactors, and intestinal enzymes (Boonthai et al., 2011). Improved water quality is associated with probiotics. The use of *Bacillus* sp. improves water quality, survival, growth performance, and health status in juvenile *P. monodon* and reduces pathogenic vibriosis in juvenile *P. monodon* (Dalmin et al., 2001) and black tiger shrimp (Rengpipat et al., 2003; Boonthai et al., 2011). We conclude that administration of a mixture of *S. phocae* PI80 and *B. subtilis* enhances disease resistance and survival in *P. monodon* juvenile and adult shrimp by producing the antagonistic compound phocaecin and stimulating the immune response. Renewal of anti-oxidant enzyme activity and total anti-oxidant status by probiotic cultures of *S. phocae* in *P. monodon* remain to be explored.

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