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Immunity enhancement in black tiger shrimp (*Penaeus monodon*) by a probiont bacterium (*Bacillus* S11)

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Abstract

Survival and growth of *Penaeus monodon* fed the probiont *Bacillus* S11 in two 90-day culture trials were increased compared with non-treated shrimp. *Bacillus* S11 also efficiently activated and increased the engulfment of foreign particles (phagocytic activity) as measured by % phagocytosis and phagocytic index (PI) in hemolymph. Phenoloxidase and antibacterial activities increased with age in all shrimp, but were even further increased by probiotic treatment. Following the second trial, after 90 days' culture with and without *Bacillus* S11 feed additives, shrimp were exposed to pathogenic, luminescent bacteria (*Vibrio harveyi*). After 10 days' exposure to *V. harveyi*, probiotic-treated shrimp had significantly greater ($p < 0.05$) survival (54.3%) compared with non-treated shrimp (35.5%). Immune responses were substantial in both treatment groups following the 10-day challenge, but were more pronounced with the probiotic-treated shrimp. The PI was significantly greater with probiotic-treated shrimp (2.7 ± 0.8) compared with controls (0.6 ± 0.3). *Bacillus* S11 provided disease protection by activating both cellular and humoral immune defenses, as well as presumably providing competitive exclusion in the shrimp's gut.

Keywords: Immunity; Probiotics; *Penaeus monodon*; Black tiger shrimp; *Bacillus* S11; *Vibrio harveyi*

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1. Introduction

Penaeid shrimp culture, especially black tiger shrimp (*Penaeus monodon*) culture, is economically important in Thailand with exports valued at US$1.6 billion in 1998 (Thai Department of Business Economics, 1999). However, Thai shrimp culture is currently suffering serious losses due to infectious diseases (Flegel et al., 1992; Lightner and Redman, 1998), including those caused by yellow-head baculovirus, systemic ectodermal and mesodermal baculovirus (white spot), and luminous bacterium (*Vibrio harveyi*; Flegel et al., 1992; Nithimathachoke et al., 1995; Ruangpan, 1998). Shrimp disease prevention and control are now priority research topics in Thailand. This research currently focuses on three areas: **Genetics** — development of disease resistant shrimp broodstock (Supungul et al., in press; Withyachumnarnkul, et al., 1998); **Immunology** — better understanding of immune response in shrimp (Adams, 1991; Itami et al., 1991; Song and Hsieh, 1994; Sung and Song, 1996; Sung et al., 1991, 1994, 1996); and **Microbiology** for development of effective shrimp probiotics (Moriarty, 1998; Rengpipat et al., 1998a).

We previously reported the isolation of a bacterial probiont, *Bacillus* S11, from healthy *P. monodon*, which reduced *P. monodon* mortality when challenged with *V. harveyi* (Rengpipat et al., 1998a). Our findings demonstrated probiotic protection, but they did not identify underlying mechanisms for this protection, nor did they characterize the shrimp's immune response. Certain microbes can induce immune responses in crustaceans. Yeast β-glucan (Sung et al., 1994, 1996), yeast zymosan (Sung et al., 1996), and dead bacterial cells (Adams, 1991; Itami et al., 1991; Sung et al., 1991, 1996) have stimulated immune responses in *P. monodon*.

Lackie (1980), Ratcliffe et al. (1985), Smith and Chisholm (1992), and Thörnqvist and Söderhäll (1997) characterized invertebrate immune systems as belonging to two broad categories, including: (1) humoral defenses such as antibacterial activity, agglutinins, cytokine-like factors, modulators, and clotting factors; and (2) cellular defenses such as hemolymph clotting, phagocytosis, nodule formation, encapsulation, and prophenoloxidase system. The relative importance of these immunological factors with *P. monodon* is still unknown. To date, invertebrate immune systems have not been shown to produce antibodies.

In our present study, based on earlier finding (Rengpipat et al., 1998a), an attempt was made to identify the affects of this probiont on *P. monodon* immunological responses. Specifically, we evaluated the immunity enhancing influence of *Bacillus* S11 on cellular defenses (total hemocytes, phagocytic activities, and phenoloxidase), and on humoral defenses (antibacterial activity).

2. Materials and methods

2.1. Bacterial strains

Bacillus S11, previously isolated from the gastrointestinal tract of *P. monodon* broodstock caught in the Gulf of Thailand in our laboratory, demonstrated effective

probiotic protection with *P. monodon* (Rengpipat et al., 1998a). *Bacillus* S11 was cultured in Tryptic soy broth (Difco) with a shaking incubator at 200 rpm, 30°C and stored on Tryptic soy agar. Cell culture after 24 h was suspended in sterile, normal saline solution before use after washing and centrifuging three times at 8,000 rpm, 4°C for 15 min. Culture purity and identity were routinely checked during this investigation.

V. harveyi D331 and *V. harveyi* 1526 cultures were provided by the Shrimp Culture Research Center, Charoen Pokphand Feedmill, Samutsakorn, Thailand. We reconfirmed the identity of *V. harveyi* following procedures described by Baumann and Schubert (1984). Both strains were isolated from *P. monodon* that died of luminescent disease. We used *V. harveyi* D331 for evaluating antibacterial activity in shrimp plasma, and for inducing infection during challenge trials in Experiment I. In Experiment II, we used *V. harveyi* 1526 to induce infection during challenge trials. Both *Vibrio* strains were cultured in Tryptic soy broth and agar with 2% NaCl (w/v).

2.2. Shrimp feed preparation

Shrimp were fed a commercial, formulated diet purchased from Grobest, Thailand. Fresh *Bacillus* S11 cells were thoroughly mixed with this feed at 1:3 (wet weight), and the mixture was spread out and dried in an oven for 1–2 h at 37°C. Feed was then stored in clean, plastic bags at 4°C until used. Shrimp feed was prepared twice weekly. Each batch was analyzed for *Bacillus* S11 and total bacterial count.

2.3. Experiment I

P. monodon were obtained from a shrimp farm in Chantaburi Province, Thailand and grew to 6–7 g after 60 days' culture in an aerated, biofiltered-recirculating water system at 30°C and 26% salinity (Menasveta et al., 1991). Shrimp age was ~ PL-60 at the first day of culture. Shrimp were placed in 150-l plastic tanks with round bottoms. Tanks were covered to reduce light intensity, and shrimp were acclimatized for 1 week before trials began. Two treatments consisted of Control, fed with unaltered shrimp diet, and Probiotic, fed with probiotic supplemented shrimp diet. Each treatment was replicated using eight tanks, each containing 20 shrimp. Shrimp were fed three times daily at 6% body weight per day. The following measurements were made every 30 days for 90 days: shrimp live weight and survival; total bacteria, *Bacillus* S11, and *Vibrio* spp. counts from rearing tank water, shrimp intestine, and feces; and immune indexes from shrimp hemolymph and plasma.

2.4. Experiment II

P. monodon were obtained from a shrimp farm in Chachoengsao Province, Thailand and grew to 0.1–0.3 g in the same system used in Experiment I. Shrimp age was ~PL-10 at the first day of culture. Shrimp were then placed in 400-l concrete tanks with flat bottoms. Tanks were covered and shrimp were acclimatized for 1 week before trials began. Two treatment groups consisted of Control, fed with unaltered shrimp diet, and Probiotic, fed with probiotic supplemented shrimp diet. Each treatment was repli-

cated using three tanks, each containing 40 shrimp. Shrimp were fed three times daily at 10% body weight per day. The following measurements were made every 30 days for 90 days: shrimp live weight and survival; total bacteria, *Bacillus* S11, and *Vibrio* spp. counts from rearing tank water and shrimp intestine. Immune indexes from shrimp hemolymph and plasma were determined on day 90 and after 10 days of challenging by *V. harveyi* 1526.

Water samples were collected weekly and monthly from the center of each tank, along with ~200 mg of shrimp feces (Experiment I only), and live shrimp for bacterial determinations. Weekly water quality measurements included; ammonium, nitrite, phosphate, temperature, dissolved oxygen, pH and salinity as described by Strickland and Parsons (1972). Commercial test kits from Merck, Germany were used for inorganic measurements. Shrimp were dissected using sterilized surgical scissors to remove intestines for microbial enumeration and identification. Bacterial determinations were made using serial dilution in NSS, followed by plating on Tryptic soy agar (with 2% w/v NaCl), and Thiosulfate citrate bile salt sucrose agar (TCBS, Difco). After 24–48 h of incubation at 37°C, colonies were counted. Microbial strains were re-examined using Gram staining, spore staining, and selected biochemical tests as described by Baumann and Schubert (1984) and Claus and Berkeley (1986). The effects of *Bacillus* S11 on shrimp growth and survival, and on *V. harveyi* 1526 resistance were evaluated using analysis of variance (ANOVA) and Duncan's multiple range tests (Statistical Analysis System, 1983).

2.5. Hemolymph collection

Hemolymph from each shrimp was collected from the ventral-sinus cavity using a 26-gauge needle and 1-ml syringe containing anticoagulant solution.

2.6. Total hemocyte count

Hemolymph (0.1 ml) was collected from three randomly selected shrimp per treatment using 0.4-ml modified KC-199 medium (K-199 medium plus HEPES 2.38 g l^{-1}, supplemented with 5% L-cysteine; Itami et al., 1994) as an anticoagulant solution. After they were mixed gently, hemocytes were counted and calculated as cells ml^{-1} using a hemocytometer with light microscope at 400 × magnification.

2.7. Phagocytic activity assay

Hemolymph (0.1 ml) was collected and mixed with 0.4-ml modified KC-199 medium as noted above (modified from Itami et al.,1994). Hemocytes were separated from hemolymph and washed with modified K-199 medium by centrifugation at 2500 rpm for 10 min at 4°C. Hemocytes (~10^7 cells ml^{-1}; 0.1 ml) were mixed with 0.1 ml of latex beads (~10^8 beads ml^{-1}, particle diameter 1.094 μ, Sigma) in modified K-199 medium on a clean glass slide. The mixture was incubated in a moisture chamber at room temperature for 30 min. After incubation, hemocytes were fixed with 2.5% glutaraldehyde. Slides were washed with modified K-199 medium to remove non-adher-

ent hemocytes, air-dried, and stained with Diff-Quick stain (Weeks-Perkins et al., 1995). Slides were mounted using permount. Numbers of ingested beads and numbers of phagocytizing cells were counted from any 200 cells observed using a light microscope at 1,000 × magnification. Percentage phagocytosis, phagocytic index (PI) and average number of the beads ingested per cell (ABPC) were calculated as follows:

Percentage phagocytosis = (no. of cells ingesting beads/no. of cells observed) × 100 (Weeks-Perkins et al., 1995).
PI = (no. of cells ingesting beads/no. of cells observed) × (no. of beads ingested/no. of cells observed) × 100 (Itami et al., 1994).
ABPC = no. of beads ingested/no. of cells ingesting beads (Itami et al., 1994).

2.8. Hemocyte lysate supernatant preparation (HLS)

HLS was prepared using modified techniques (Smith and Söderhäll, 1991). Briefly, shrimp hemolymph (0.1 ml) was collected and mixed with 0.4 ml modified KC-199 medium, then centrifuged at 2500 rpm for 10 min at 4°C. Hemocytes were washed and collected in ice cold cacodylate (CAC) buffer at pH 7.0 (0.01 M sodium cacodylate, 0.45 M NaCl, 10 mM $CaCl_2$, 26 mM $MgCl_2$). This suspension was homogenized with a sonicator equipped with a microtip (output 5, duty cycle 50%) for 3 s, and centrifuged at 15,000 rpm for 20 min at 4°C. The resultant HLS was used immediately as an enzyme source.

2.9. Phenoloxidase activity assay

Phenoloxidase activity in HLS was estimated spectrophotometrically using L-3, 4-dihydroxyphenylalanine (L-DOPA; Sigma) as substrate (Söderhäll, 1981), and trypsin (Sigma, cat. no. T0646) as elicitor following the method described by Smith and Söderhäll (1991). A total of 200 μl of HLS was incubated with 200 μl of 0.1% trypsin in CAC buffer at room temperature for 30 min, and then 200 μl of L-DOPA (0.3% in CAC buffer) was added. Each reaction mixture was further diluted with 600 μl of CAC buffer, mixed and optic density was measured at a 490 nm. Absorbance measurements were made against a blank consisting of CAC buffer, L-DOPA and elicitor to control for spontaneous oxidation of the substrate alone. One unit of enzyme activity was defined as an increase in absorbance of 0.001/min/mg protein (Söderhäll and Unestam, 1979). Protein content in HLS was measured via the Bradford method (Bradford, 1976), using bovine serum albumin as a standard protein.

2.10. Antibacterial activity

V. harveyi D331 was cultured in brain heart infusion broth with 2% NaCl (w/v) overnight at 37°C. The concentration was adjusted to $\sim 10^4$ CFU ml^{-1}, collected and washed in 2% (w/v) sterile saline by centrifuging at 10,000 rpm for 15 min at 4°C. Shrimp hemolymph (0.1 ml) was collected and mixed with 1.4-ml of ice cold, sterile Van Harrevald's salt solution (VHS; Van Harrevald, 1936), and then centrifuged at 11,000 rpm for 10 min at 4°C. Plasma was collected and sterilized by filtration (millipore membrane filter, 0.45 μm pore size). *V. harveyi* D331 concentrate and shrimp

plasma (0.1 ml each) were mixed in sterile test tubes and incubated for 1 h at 37°C. Three 50 μl aliquots were taken from each tube and spread on TCBS agar plates to estimate bacterial numbers (CFU). Plates were incubated at 37°C for 24 h. Positive controls of *V. harveyi* D331 in their respective saline were incubated with VHS (Adams, 1991).

2.11. Percentage inhibition

Percentage inhibition was calculated where; % inhibition = 100 − (mean CFU sample/mean CFU positive control) × 100 (Adams, 1991). Immune indexes were evaluated at $p < 0.05$ for significant values by using ANOVA and Duncan's multiple range tests (Statistical analysis System, 1983).

2.12. Challenge tests

After 90 days, shrimp in Experiments I and II were challenged with *V. harveyi* which had been cultured and maintained using Tryptic soy broth and agar with 2% NaCl (w/v). With Experiment I, all shrimp were immersed in a *V. harveyi* D331 suspension at $\sim 10^7$ CFU ml^{-1} (modified from Rengpipat et al. 1998a) according to Austin et al. (1995). This was followed by a second immersion of $\sim 10^7$ CFU ml^{-1} after 7 days. Since no shrimp death occurred during these 14 days, we then added a suspension of *V. harveyi* 1526 at $\sim 10^8$ CFU ml^{-1} to each tank. All shrimp in both control and treatment died soon thereafter (data not shown).

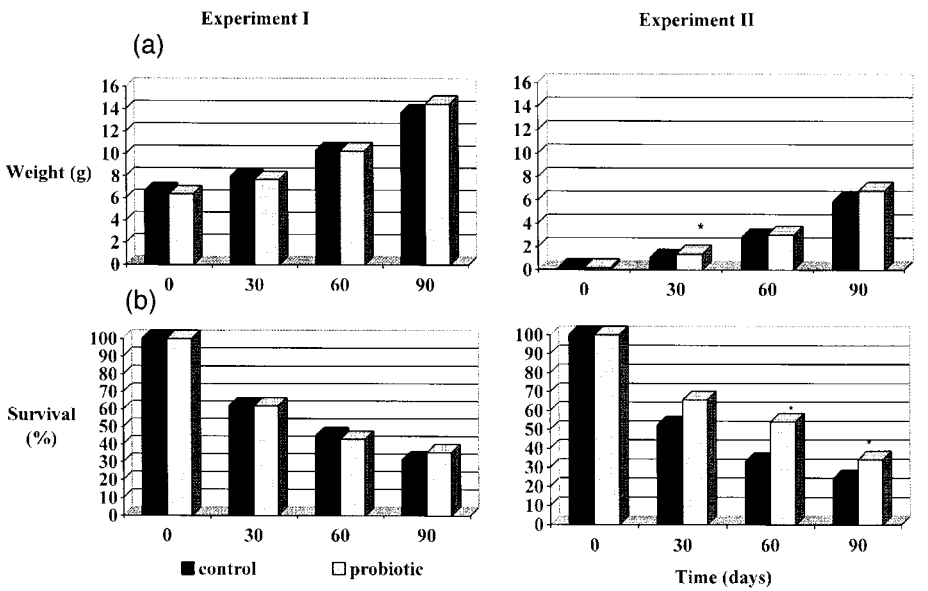


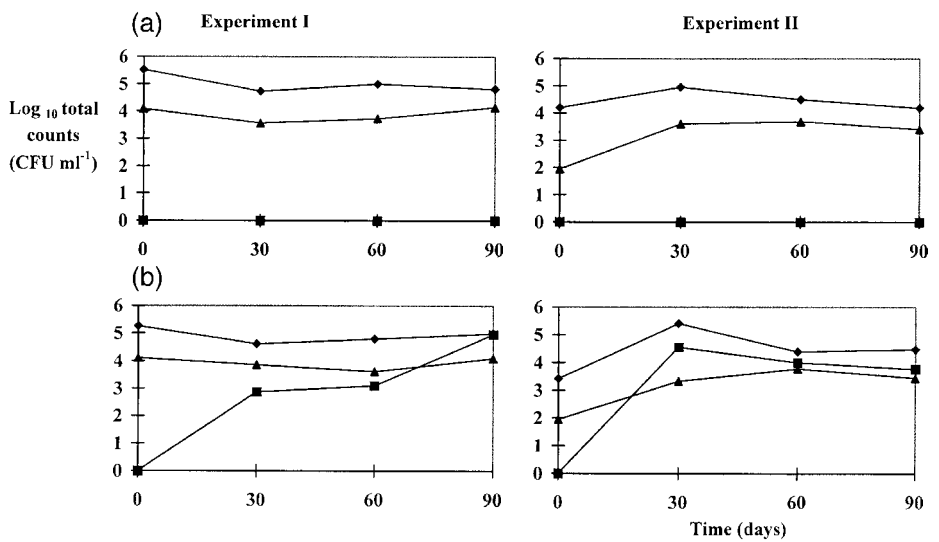
Fig. 1. Average weights and survivals of *P. monodon* during 90 days of culture when provided with two feeds: with and without probiotic supplements. Results from both Experiments I and II are shown. * Significantly different at $p < 0.05$.

In Experiment II, 30 shrimp from a control group and 32 shrimp from the probiotic treatment were immersed in tanks containing *V. harveyi* 1526 at $\sim 10^7$ CFU ml^{-1}. Three concrete tanks (10 or 11 shrimp per tank) per treatment were used. A second *V. harveyi* addition was made at the same concentration on the fourth day. During immersion tests, water and shrimp were collected and examined every 2 days from each tank. After 10 days of the challenge test, shrimp in each group were dissected by sterile surgical scissors and examined microbiologically as described above. Shrimp survival was determined for each replicate. *V. harveyi* culture isolated from 10 shrimp hepatopancreas and intestines from each replicate were purified and identified using characteristic of green and luminescent colonies on TCBS, Gram staining, oxidase test, and motility test. These were compared with the original *V. harveyi* 1526 cultures, in order to confirm *V. harveyi* strain identity using procedures described by Baumann and Schubert (1984).

3. Results

3.1. Shrimp feed preparation

Bacillus spp. was the predominant bacteria found in unaltered feeds based on agar plate counts, generally at concentrations of $\sim 10^2$ CFU g^{-1}. Our altered feed, supple-



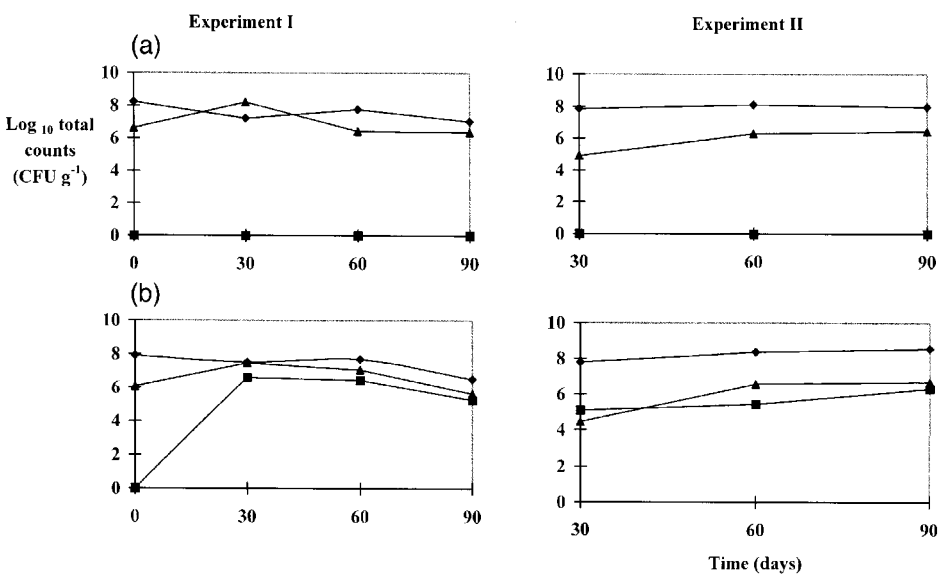
Total bacteria (-◆-), *Bacillus* S11 (-■-) and *Vibrio* spp. (-▲-)

Fig. 2. Bacterial counts in rearing tank waters of Experiments I and II during 90 days of feeding with: (a) control and (b) probiotic feeds. All values are means of eight replicates for Experiment I and three replicates for Experiment II, per treatment.

mented with probiont *Bacillus* S11 typically had 4.69×10^{10} CFU g^{-1} on the first day of storage, and 1.39×10^{10} CFU g^{-1} after 1 week storage at 4°C. Feed stored at 4°C for up to 90 days had little further decrease in *Bacillus* S11 concentrations ($\sim 10^{10}$ CFU g^{-1}).

3.2. Water quality

There were no obvious effects of *Bacillus* S11 on water quality in probiotic treatments during either Experiments I or II. With Experiment I, water quality values were as follows: dissolved oxygen 5.0–7.2 mg l^{-1}, pH 7.4–8.1, temperature 25.6–31.7°C, and salinity 26 ppt. Other values were; ammonium 0.0–0.56 mg l^{-1}, nitrite 0.1–2.5 mg l^{-1}, and phosphate 8.0–26.3 mg l^{-1}. In Experiment II, water quality values were; dissolved oxygen 5.2–7.5 mg l^{-1}, pH 7.6–8.2, temperature 24.0–27.5°C, salinity 26–30 ppt, ammonium 0.0–0.42 mg l^{-1}, nitrite 0.08–0.33 mg l^{-1}, and phosphate 3.3–10.0 mg l^{-1}. All of these water quality parameters were considered safe for shrimp culture, and there were no significant differences ($p > 0.05$) between corresponding mean values for control and probiotic treatments. Although phosphate values were initially elevated, and further increased during our trials, these concentrations would not



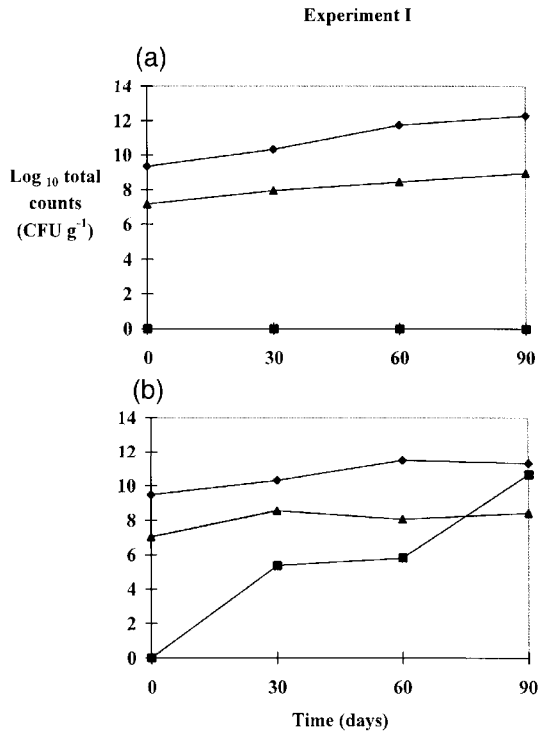
Total bacteria (-◆-), *Bacillus* S11 (-■-) and *Vibrio* spp. (-▲-)

Fig. 3. Bacterial counts in shrimp intestines of Experiments I and II during 90 days of feeding with: (a) control, and (b) probiotic feeds. All values are means of eight replicates for Experiment I and three replicates for Experiment II, per treatment.

have harmed the shrimp. The main source of phosphate was from the feed. There were little or no phosphate losses from the recirculating water system.

3.3. Shrimp growth

Mean shrimp weights in Experiment I after 90 days were 14.5 ± 1.7 and 13.7 ± 2.0 g in the probiotic and control groups, respectively, while shrimp survivals were 35.6 ± 7.8% and 31.3 ± 15.3%, respectively. These mean values were not significantly different ($p > 0.05$, Fig. 1). In Experiment II, mean shrimp weights after 90 days were 6.9 ± 0.9 and 5.9 ± 1.0 g, respectively, with no significant difference (Fig. 1). There was, however, a significant difference in mean weights after 30 days' culture. Probiotic-treated shrimp also had significantly greater survival (34.2 ± 3.6%) compared with controls (24.2 ± 5.1%). In both experiments, cannibalism associated with clear water apparently caused low survival (Rengpipat et al., 1998a). Overall, though, probiotic-treated *P. monodon* appeared healthier and were more active than those in the control group.



Total bacteria (-◆-), *Bacillus* S11 (-■-) and *Vibrio* spp. (-▲-)

Fig. 4. Bacterial counts in shrimp feces of Experiment I during 90 days of feeding with: (a) control, and (b) probiotic feeds. All values are means of eight replicates per treatment.

3.4. Bacterial patterns

Total bacterial counts in rearing tank waters of Experiment I ranged from 6.4×10^4 to 1.0×10^5 and 4.1×10^4 to 1.8×10^5 CFU ml^{-1} in control and probiotic groups, respectively (Fig. 2). *Vibrio* spp. concentrations were also not significantly different between treatment and control tanks. *Bacillus* S11 was only found in rearing water of probiotic treatments, and ranged from 7.5×10^2 CFU ml^{-1} on day 30 to 9.2×10^4 CFU l^{-1} by day 90. In Experiment II, total bacterial counts were $1.6–8.9 \times 10^4$ and 2.6×10^3 to 2.5×10^5 CFU ml^{-1} in rearing tank waters of control and probiotic groups, respectively (Fig. 2). There were no significant differences in mean values of total bacteria or for *Vibrio* spp. in rearing water. *Bacillus* S11 ($\sim 10^4$ CFU ml^{-1}) was only detected in rearing tank waters of the probiotic group (Fig. 2).

Total bacterial counts of 1.1×10^7 to 1.7×10^8 and 3.3×10^6 to 8.2×10^7 CFU g^{-1} were found in control and probiotic shrimp intestines, respectively in Experiment I (Fig. 3). *Vibrio* spp. ranged from 2.4×10^6 to 1.6×10^8 CFU g^{-1} and 4.6×10^5 to 2.9×10^7 CFU g^{-1} in control and probiotic shrimp intestines, respectively. *Bacillus* S11 was only found in probiotic shrimp intestines at $\sim 10^6$ CFU g^{-1} after 30 days' culture, but not in intestines of control shrimp. *Vibrio* spp. concentrations decreased 2-log cycles after 1-month culture with probiotic treatment. In Experiment II, total bacterial counts in shrimp intestines were 7.2×10^7 to 1.3×10^8 CFU g^{-1} for both treatment groups during the last 2 months of culture (Fig. 3). *Bacillus* S11 ($\sim 10^5$ CFU g^{-1}) was found only in shrimp fed probiotic feeds.

Total bacterial counts in shrimp feces of Experiment I ranged from 2.3×10^9 to 1.9×10^{12} and 3.0×10^9 to 3.1×10^{11} CFU g^{-1} in control and probiotic groups,

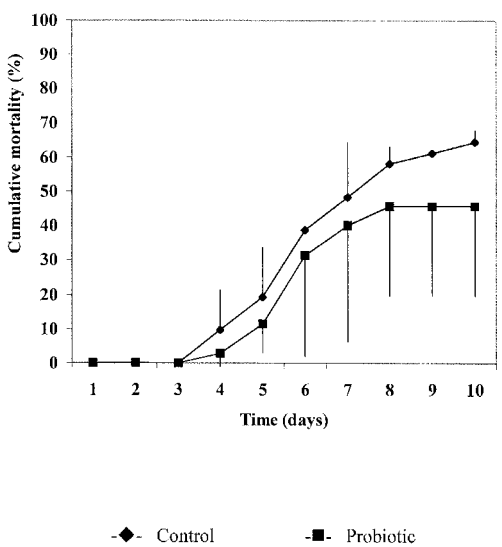


Fig. 5. Average cumulative mortality of *P. monodon* in Experiment II as a function of time during the 10-day challenge tests with *V. harveyi* 1526. All values are means of three replicates per treatment. No mortality occurred in a control group without *V. harveyi* 1526. Vertical bars indicate mean ± S.D.

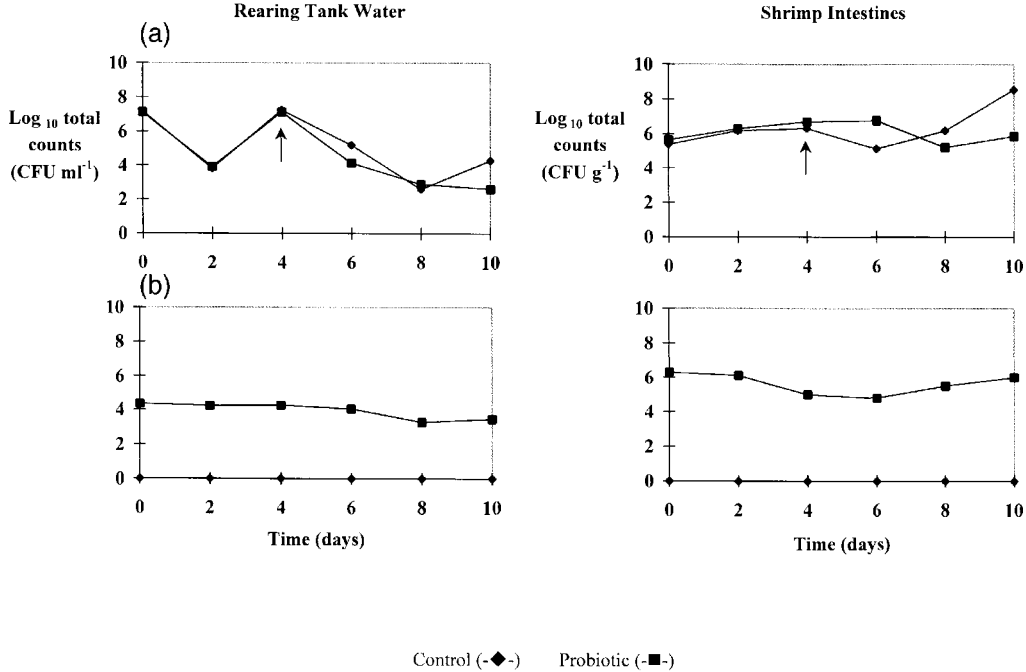


Fig. 6. Bacterial counts of (a) *V. harveyi* 1526 and (b) *Bacillus* S11 in rearing tank waters, and in *P. monodon* intestines during 10 days' challenge with *V. harveyi* 1526 at $\sim 10^7$ CFU ml^{-1} by immersion (↑ = second *V. harveyi* booster innoculation). All challenge test results were for Experiment II only. All values are means of three replicates per treatment.

respectively (Fig. 4). *Vibrio* spp. ($\sim 10^7$ to 10^8 CFU g^{-1}) was detected in shrimp feces from both groups of Experiment I. *Bacillus* S11 at $\sim 4.4 \times 10^{10}$ CFU g^{-1} was found in shrimp feces after 90 days' culture, while it was not detected in control shrimp feces.

3.5. Challenge test of Experiment II

During the 10-day challenge test of Experiment II, cumulative shrimp mortality in the probiotic treatment (45.7%) was less than that of the control group (64.5%; Fig. 5). Survival was also more variable with the probiotic group. After adding *V. harveyi* to culture tanks, 1.55×10^7 and 1.29×10^7 CFU ml^{-1} of this strain were detected in rearing water of control and probiotic group, respectively (Fig. 6). Four days later, *V. harveyi* in rearing water in both treatments decreased to $\sim 10^3$ CFU ml^{-1}, and a second addition was made to all tanks to increase *V. harveyi* to $\sim 10^7$ CFU ml^{-1} again. *V. harveyi* in the rearing tank water of the probiotic group was lower than that of the

Table 1
Mean immunity index values of control- and probiotic-treated *P. monodon* during 90 days of culture in Experiment I

Immunity indexes	Duration of shrimp culture (days)	Means ± S.D.	
		Control	Probiotic
Total hemocytes[1]	30	2.6 ± 0.3^a	4.3 ± 2.7^a
(1×10^7cell ml^{-1})	60	2.9 ± 1.0^a	5.5 ± 1.5^a
	90	2.0 ± 1.3^a	4.6 ± 1.6^a
Phagocytic activity[1]			
% phagocytosis	30	3.2 ± 1.7^a	4.4 ± 4.0^b
	60	7.7 ± 2.4^a	$16.3 \pm 0.8^{a*}$
	90	5.8 ± 0.4^a	6.8 ± 0.4^b
Phagocytic index	30	0.2 ± 0.2^b	0.5 ± 0.7^b
	60	3.3 ± 1.8^a	$12.8 \pm 4.3^{a*}$
	90	1.1 ± 0.0^{ab}	1.7 ± 0.6^b
ABPC	30	1.6 ± 0.2^a	2.1 ± 0.9^a
	60	5.9 ± 2.5^a	4.9 ± 1.6^a
	90	2.7 ± 0.1^a	3.6 ± 0.5^a
Phenoloxidase[2]	30	33.7 ± 17.0^a	39.0 ± 25.1^b
(units/min/mg Protein)	60	763.8 ± 639.4^a	750.6 ± 97.4^a
	90	624.1 ± 516.2^a	767.3 ± 258.0^a
Antibacterial activity[3]	30	0.0 ± 0.0^b	47.6 ± 46.7^a
(% inhibition)	60	88.5 ± 7.4^a	96.0 ± 3.3^a
	90	71.8 ± 17.4^a	97.3 ± 1.0^a

Means not sharing a common superscript letter between column values for each indexes differ significantly ($p < 0.05$).
ABPC = The average numbers of beads ingested per cell.
* Indicates significant difference ($p < 0.05$) between control and probiotic treatment means for a given row.
[1] $n = 3$.
[2] $n = 5$.
[3] $n = 6$.

control on some days. *Bacillus* S11 was not detected in rearing water and shrimp intestines of the control group, but it was $\sim 10^3$–10^4 CFU ml^{-1} in rearing water and $\sim 10^5$–10^6 CFU g^{-1} in shrimp intestines of the probiotic group (Fig. 6). *V. harveyi* 1526 was detected in shrimp intestines of control group from 2.1×10^5 up to 3.4×10^8 CFU g^{-1} on day 10, whereas it was $\sim 10^6$ CFU g^{-1} in probiotic intestines during the 10-day challenge test. *Bacillus* S11 survived in *P. monodon* intestines and apparently interfered with *V. harveyi* growth as shown in Fig. 6a and b.

3.6. Immunity indexes

All probiotic-treated shrimp had greater immunity indexes than those of the controls (Table 1). Significant differences ($p < 0.05$) occurred between control and probiotic treatment means with % phagocytosis and the PI by day 60. All three PIs of both control and probiotic treatments had much greater mean values on day 60 compared with day 30, with subsequent decreases by day 90. Total hemocyte means showed this same pattern, as did phenoloxidase control shrimp. Except for total hemocyte means, immunity indexes tended to increase with age between day 30 and day 90 for both controls and probiotic-treated shrimp. Our small sample size could account for fewer significant differences between means.

After 90 days' culture in Experiment II, but preceding the *V. harveyi* challenge, probiotic-treated shrimp all had greater mean immunity indexes compared with controls (Table 2). Probiotic-treated shrimp had significantly greater ($p < 0.05$) mean total hemocyte and phenoloxidase activities at that time. After 10 days' challenge with *V.*

Table 2
Mean immunity index values of control- and probiotic-treated *P. monodon* before and after 10 days' challenge with *V. harveyi* 1526 in Experiment II

Immunity indexes	Means ± S.D.			
	Before		After	
	Control	Probiotic	Control	Probiotic
Total hemocytes[1] (1×10^7 cell ml^{-1})	1.4 ± 0.6^{b}	2.6 ± 0.7^{a}	1.1 ± 0.5^{b}	1.1 ± 0.2^{b}
Phagocytic activity[1]				
% phagocytosis	1.0 ± 0.5^{c}	2.2 ± 1.0^{c}	6.0 ± 1.8^{b}	10.5 ± 1.8^{a}
Phagocytic index	0.0 ± 0.0^{c}	0.1 ± 0.1^{c}	0.6 ± 0.3^{b}	2.7 ± 0.8^{a}
ABPC	1.6 ± 0.5^{a}	2.0 ± 0.4^{a}	1.7 ± 0.3^{a}	2.5 ± 0.3^{a}
Phenoloxidase[2] (units/min/mg Protein)	10.3 ± 9.0^{b}	41.0 ± 10.1^{a}	7.7 ± 1.0^{b}	24.7 ± 12.6^{ab}
Antibacterial activity[3] (% inhibition)	17.9 ± 28.1^{c}	32.4 ± 29.1^{bc}	70.5 ± 15^{ab}	87.4 ± 9.3^{a}

Means not sharing a common superscript letter between row values differ significantly ($p < 0.05$).
ABPC = The average numbers of beads ingested per cell.
[1] $n = 3$.
[2] $n = 5$.
[3] $n = 6$.

harveyi, all immunity index means of both control and probiotic treatments increased substantially, except for total hemocytes, which decreased. These immune responses were generally more pronounced with probiotic-treated shrimp.

4. Discussion

Growth and survival differences between *Bacillus* S11-treated and non-treated *P. monodon* in earlier studies (Rengpipat et al., 1998a,b) were more pronounced than during our present study. While *P. monodon* growth and survival was greater for shrimp treated with probiotic bacteria in our present study, they were not significantly different with all treatments (Fig. 1). Reasons for reduced significance levels could be related to the performance of our culture system. We typically experience much slower growth with our recirculated water system in comparison to an outdoor pond culture. Our recirculation systems had clear water with fine particle filtration. This may have removed important nutritional components, such as those reported by Moss et al. (1992). Although the observed high phosphate concentrations were not toxic, they could indicate a system imbalance, which may have negatively affected all shrimp performance.

Recently, three approaches have been used to improve shrimp health and yields. First, use of specific disease-resistant shrimp (SPF). Second, vaccination or immunostimulation of shrimp to promote immune response. Lastly, probiotic use to stimulate immunity and to exclude pathogens. In addition, stress reduction is used to improve disease resistance in shrimp. Stress caused by poor water quality, for example, can make shrimp susceptible to less virulent pathogens. In these cases, the pathogens are often considered secondary infections (Song et al., 1993). Since shrimp possess a non-specific immune response (Anderson, 1992), vaccination or immunostimulation may provide only short-term protection against specific pathogens (Sung and Song, 1996; Sung et al., 1996). Effective probiotic treatments, on the other hand, may provide broader-spectrum and greater non-specific disease protection as a result of both serological immunity enhancement and competitive exclusion in shrimp guts.

Our findings show the presence of viable *Bacillus* S11 in intestines and feces of shrimp fed this probiont (Figs. 3 and 4). Competitive exclusion most likely occurred in this case (Fig. 4b). Water quality is not effected by probiotic feed additives (Rengpipat et al.1998a), but shrimp health and prophylactic functions can be enhanced (Austin et al., 1992, 1995; Rengpipat et al., 1998a,b; Phianphak et al., 1999). Our findings have also confirmed that probiotics should be fed starting at an early age for optimal improvement of indigenous gut microflora (Rengpipat et al., 1998b). At the same time, probionts will proliferate in rearing water thus providing a better environment for shrimp by reducing the level of certain of pathogens in the culture water (Moriarty, 1998). *Bacillus* S11, a saprophytic strain appears harmless to shrimp and culture system, while providing greater shrimp survival during normal culture and following disease challenge by luminescent bacterial challenges (Rengpipat et al., 1998a). This suggests that probiotic treatment is an effective alternative for enhancing shrimp health.

This study demonstrated promising results for immune response stimulation in *P. monodon*. Only a few other published reports concern immunity enhancement with

penaeid shrimp and other crustaceans. These include phenoloxidase activity in *P. monodon* (Sung et al., 1994), anti-*Escherichia coli* activity (Sung et al., 1996), bactericidins in *P. monodon* (Adams, 1991), phagocytic activity in *P. japonicus* (Itami et al., 1994), phagocytic hemocytes (%) and proPO system in *P. californiensis* (Vargas-Albores, 1995), phagocytosis in fresh water crayfish (McKay and Jenkin, 1970), bactericidin in West Indian spiny lobster (Weinheimer et al., 1969), and phagocytic hemocytes in American lobster (Paterson and Stewart, 1974). In this study, greatly elevated phenoloxidase and antibacterial activity in *P. monodon* was detected in hemolymph with older shrimp (> PL 90; Table 1). One possible explanation is that phenoloxidase was produced from the activation of the prophenoloxidase (proPO system) as a cascade phenomena with several steps for function performance (Söderhäll et al., 1990). Antibacterial activity may have developed gradually, and was thus more fully expressed in larger shrimp. Meanwhile, antibacterial activity should be elicited when shrimp are exposed to foreign bacteria. Shrimp may need some time to adapt to or respond immunologically to their changing environment. Average antibacterial activity in shrimp plasma with Experiment I (Table 1) was undetectable in control groups at an early age, and relatively low in Experiment II after 90 days' culture (Table 2). There was also much variability. Antibacterial activity in Experiment II after 90 days was $17.9 \pm 28.1\%$ inhibition with range of 0–56.5%. No antibacterial activity was detected in some young shrimp. Nevertheless, phagocytic hemocytes are the primary, nonspecific defense mechanism against invasion of pathogenic organisms in invertebrates (Robohm, 1984; Olivier et al., 1988), and actively function at all shrimp ages (Table 1). They are used to determine shrimp health and for evaluation of crustacean cellular immune response (Johansson, 1995). Significantly greater % phagocytosis occurred with the probiotic group, which tended to increase with age for both groups (Table 1). Percent phagocytosis significantly increased in both groups after the challenge test (Table 2), which strongly confirmed the presence of circulating phagocytic hemocytes in *P. monodon* hemolymph that acted as a major nonspecific immune defense for all ages of shrimp. Cell types of hemocytes, hyaline, semigranulated or granulated cells that act as a phagocytic function are still not known (Johansson and Söderhäll, 1989; Hose et al., 1990; Itami et al., 1998).

In Experiment II, enhancement of % phagocytosis and antibacterial activity clearly increased after infection in both control and probiotic groups (Table 2). Total hemocytes decreased after infection in both groups with a significant decrease in the probiotic treatment ($p < 0.05$). Similar rapid and marked reduction in circulating hemocyte numbers, in freshwater crayfish (*Astacus astacus*) and shore crabs (*Carcinus maenas*) have also been reported, which indicated initiation of cellular defense reactions (Smith and Söderhäll, 1983). We observed significantly increased % phagocytosis after *Vibrio* infection in both control and probiotic groups as well (Table 2).

All immunity indexes in *Bacillus* S11-treated *P. monodon* were higher than those of non-treated groups with the significant differences ($p < 0.05$) of % phagocytosis and PI (Table 1), and total hemocytes and phenoloxidase (Table 2). Since *Bacillus* S11 is a long-term resident in probiotic-treated shrimp guts it should provide a longer-term immunostimulant for shrimp compared with glucan or other such immunostimulants (Sung et al. 1994). *Bacillus* S11 surface antigens, or their metabolites might act as

immunogens for shrimp immune defense. *Bacillus* S11 cell wall peptidoglycan might elicit an immune function in shrimps (Itami et al., 1998) by acting on granulocytes for higher phagocytic activity. As a result, the phagocytic hemocytes of the probiotic-treated shrimp engulfed foreign particles more aggressively. This result supports the previous finding of Itami et al. (1998) where ABPC increased phagocytic hemocytes after shrimp immunity induction by peptidoglycan. Also, some dead *Bacillus* S11 or their spores might act as a "Bacterin", which is a vaccine prepared from dead cell of bacteria that functions as a prophylaxis and probiotic (Sung et al., 1991).

When probiotic and control shrimp were challenged with *V. harveyi* both groups had a significant increase ($p < 0.05$) in the PI, but the probiotic-treated shrimp had significantly greater PI increase compared with controls (Table 2).

In summary, the probiont *Bacillus* S11 provided both cellular and humoral immune defense responses in *P. monodon*. These dual defense responses were elicited by this single immunogen, which resided in the shrimp's gut. This residency presumably further protected *P. monodon* against pathogenic bacterial infection by competitive exclusion. The relative importance of these defenses is still unknown, but they collectively conferred bacterial disease protection. It also appears that the use of this probiont was most effective when it was provided at an early age and continued during culture. Probiotic and other immunostimulant use are more desirable and environmentally benign compared to the use of antibiotics and chemicals.

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