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The effect of *Bacillus* spp. bacteria used as probiotics on digestive enzyme activity, survival and growth in the Indian white shrimp *Fenneropenaeus indicus*

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Abstract

This study examined the effects of a commercial *Bacillus* probiotic on the digestive enzyme activity, survival and growth of *Fenneropenaeus indicus* at various ontogenetic stages in three separate experiments: (1) Nauplius_{1–2} to Zoea₃, which were exposed to probiotic added directly to the water; (2) Mysis₁ to PL₁₄ in tanks, which were exposed to the probiotic either through adding it directly to the water or by feeding shrimp with probiotic-enriched *Artemia*; (3) postlarval shrimp reared in earthen ponds during the farming stages (PL₃₀ to PL₁₂₀), which were exposed to probiotic added to the water. The counts of *Bacillus* bacteria in the digestive tract in all treatments were significantly ($P < 0.05$) higher than in controls (no *Bacillus* bacteria were detected in any controls), although total bacterial counts were not significantly different among treatments and controls. Colonization rates of shrimp digestive tracts by *Bacillus* bacteria were very low in all treatments in earthen ponds. In most treatments, the specific activities of amylase, total protease, and lipase were significantly higher ($P < 0.05$) in shrimp to which probiotic had been administered, and shrimp that had received probiotic exhibited significant ($P < 0.05$) increases in both survival (11–17% higher) and wet weight (8–22% higher) as compared to controls. Shrimp fed probiotic-enriched *Artemia* had significantly ($P < 0.05$) higher *Bacillus* counts than did shrimp administered probiotic in the water, but growth and survival were not significantly different between the two modes of administration. Where probiotic was administered during both the hatchery stages (Nauplius_{1–2} through PL₃₀) and the farming stages, the feed conversion ratio, specific growth rate, and final production were slightly, but significantly ($P < 0.05$), higher in shrimp receiving the probiotic than in control shrimp which had received no probiotic. Because these improvements in growth parameters in postlarval shrimp were significant only in shrimp

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that had received the probiotic both during hatchery stages and during farming stages, it appears to be important for the shrimp to receive the probiotic in all ontogenetic stages in order for these improvements to be realized.

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Keywords: *Bacillus*; Probiotic; Indian white shrimp; *Fenneropenaeus indicus*; Growth; Survival; Digestive enzyme activity

1. Introduction

The use of probiotics in the culture of aquatic organisms is increasing with the demand for more environment-friendly aquaculture practices (Gate-soupe, 1999). A probiotic is generally defined as a live microbial food supplement which improves the balance of the host animal's intestinal flora (Fuller, 1989). However in aquaculture, probiotics can be administered either as a food supplement or as an additive to the water (Moriarty, 1998). Probiotics in aquaculture have been shown to have several modes of action: competitive exclusion of pathogenic bacteria through the production of inhibitory compounds; improvement of water quality; enhancement of immune response of host species; and enhancement of nutrition of host species through the production of supplemental digestive enzymes (Thompson et al., 1999; Verschuere et al., 2000).

Because *Bacillus* bacteria secrete many exoenzymes (Moriarty, 1996, 1998), these bacteria have been used widely as putative probiotics. Studies have shown that when these bacteria were administered as probiotics in the shrimp *Penaeus monodon*, growth and survival were improved and immunity was enhanced (Rengpipat et al., 1998a,b, 2000). However, the nutritional effects of probiont bacteria, especially the effects of the bacteria on digestive enzyme activity, have not been evaluated in aquaculture. The present study examines the effect of *Bacillus* probionts on digestive enzyme activity, survival and growth in the shrimp *Fenneropenaeus indicus*.

2. Materials and methods

2.1. Rearing of shrimp

Three separate experiments were conducted to examine the effect of probiotics administered to the Indian white shrimp *F. indicus* H. Milne-Edwards,

1837. All treatments and controls were repeated in triplicate. Larvae and postlarvae were obtained from eyestalk-ablated spawners (average weight 40 g). Shrimp nauplii were reared in aerated tanks with 31–32‰ salinity natural seawater supplemented with a mixture of the microalgae *Chaetoceros* and *Tetraselmis*, which was added daily at a rate of 2×10^6 cells/ml. Typically, 10% of the tank water was exchanged each day. Beginning at mysis stage 1 (M₁), shrimp larvae were fed *Artemia franciscana* nauplii at a rate of 3–4 nauplii for each shrimp larva 4 times per day until mysis stage 3 (M₃) and at a rate of 8–10 nauplii for each shrimp larva 5–6 times per day from M₃ through 14 days after metamorphosis (PL₁₄). Although the microalgae were used as food primarily by early larval stages, we continued supplementing the culture tanks with microalgae throughout the hatchery stages, in order to maintain a green-water culture system. After the end of the hatchery stage (PL₃₀), postlarvae were transferred to 100 m² earthen ponds with a mean depth of 1.0 m at a rate of 20 postlarvae/m². Ponds were filled with 39.0–42.1‰ seawater (average, 41.3‰); 10% of the pond water was exchanged daily. Shrimp in ponds were fed commercial pellets containing 38–42% crude protein (Havourash Co., Boushehr, Iran) at a rate of 7–11% of shrimp body weight per day for the first month and then at 5–6% of body weight per day until harvested.

2.2. Experimental design

The commercial probiotic used in this study (Pro-texin Aquatech, Probiotics International Ltd, Somerset TA146QE, United Kingdom) contained spores of 5 species of *Bacillus* (i.e., *B. subtilis*, *B. licheniformis*, *B. polymyxa*, *B. laterosporus* and *B. circulans*). Before administration of the probiotic, spores were rehydrated to vegetative bacteria according to manufacturer's instructions (see Ziaei-Nejad, 2004, for details). In treatment P_w, the probiotic was added directly to the water at the recommended dosage. In

Experiments I and II, probiotic suspension was added to the tanks daily at a rate of $7.3 \pm 0.2 \times 10^6$ CFU of probiont/ml of culture medium; in Experiment III, $1.0 \pm 0.3 \times 10^7$ CFU/ml of pond water was added to each pond weekly (see Ziaei-Nejad, 2004). In treatment P_a, the shrimp were fed *A. franciscana* nauplii that had been enriched with probiotic: decapsulated *Artemia* cysts were hatched in water to which probiotic had been applied at a rate of $2.2 \pm 0.1 \times 10^7$ CFU of probiont/ml of culture medium; *Artemia* nauplii were harvested 10 h after hatching (see Ziaei-Nejad, 2004, for details) and then were fed to the shrimp larvae.

2.2.1. Experiment I

The effect of the probiotic on early larval stages, nauplius stage 1 and 2 (N_{1–2}) through zoea stage 3 (Z₃), was examined in Experiment I. Shrimp (*F. indicus*) were stocked in 10-l plastic tanks at a density of 1000 nauplii per tank. Water in tanks was not exchanged during the experiment (6 days). The probiotic was added directly to the tank water in treatment P_w and results were compared with those of controls (C) in which larvae were reared in water that had not received the probiotic.

2.2.2. Experiment II

The effect of the probiotic on larvae and postlarvae (M₁ through PL₁₄) was examined in Experiment II. M₁ larvae used in this study had been reared in a 300 l tank as described above with no probiotic and were transferred to 10-l plastic tanks at a stocking density of 500 larvae per tank at the start of the experiment; 10% of the tank water was exchanged each day. In treatment P_w, the probiotic was added directly to the water; in treatment P_a, the shrimp were fed probiotic-enriched *Artemia*. Results of these two treatments were compared with those from controls (C) in which no probiotic had been administered.

2.2.3. Experiment III

The effect of probiotic administered in the water to postlarval shrimp reared in earthen ponds was examined in Experiment III. In treatment P, the probiotic was added only in the farming stages (PL₃₀ to PL₁₂₀). In treatment PP, the probiotic was added to the water both in the hatchery stages (N_{1–2} to PL₃₀) and in the farming stages. Results from these treatments were

compared with those from controls (C) which had received no probiotic in either the hatchery stages or the farming stages.

2.3. Monitoring of bacteria

Water samples and digestive tract samples from shrimp and enriched *Artemia* were examined to determine counts of total bacteria and counts of the probiont *Bacillus*. Prior to dissection or homogenation, the shrimp larvae, postlarvae and *Artemia* nauplii were rinsed with sterilized distilled water, washed with 0.1% benzalkonium chloride according to Gate-soupe (1999), and then rinsed again with sterilized distilled water to removal all external bacteria. For both the shrimp and *Artemia* from Experiments I and II, whole organisms were homogenized in order to enumerate bacteria present within the digestive tract. In Experiment III, the digestive tract (foregut, hepatopancreas, and intestine) was dissected out using sterile technique and then was homogenized. All samples were diluted serially with sterilized normal saline solution (0.85% w/v NaCl). Total counts of bacteria were determined by plating on tryptic soy agar (with 1% w/v NaCl), according to Shariff et al. (2001). *Bacillus* bacteria in water samples were cultured using a surface drop technique with yeast extract agar. Digestive tract samples were cultured on *Bacillus cereus* agar (Oxoid CM617) according to the method recommended by Probiotics International Ltd. (Protexin Aquatech, Registration Dossier, unpublished pamphlet). The number of colonies on each plate was counted after incubation for 68 h at 25 °C for water samples and for 72 h at 37 °C for digestive tract samples.

2.4. Assay of enzyme activity

To measure digestive enzyme activity, samples of shrimp were collected after all larvae had reached Z₃ in Experiment I, immediately after metamorphosis (PL_{1–2}) and at PL₁₄ in Experiment II, and at PL₈₆ and PL₁₂₀ in Experiment III. All samples were collected at mid-morning (between 10.00 h and 12.00 h), following the methods of Ribeiro and Jones (2000) and Brito et al. (2001), and then were washed with cold distilled water and immediately frozen at –70 °C until enzyme assays were conducted.

As with monitoring of bacteria, whole animals were homogenized in Experiments I and II, but in Experiment III the digestive tract was dissected out and homogenized. Samples were homogenized in 9 volumes of 0.05 M Tris (hydroxy methyl) amino-methane hydrochloride buffer, pH 7.8, with 0.011 M CaCl₂ in a glass homogenizer and were centrifuged at 4800×g for 60 min at 4 °C according to the method outlined in Lovett and Felder (1990). The supernatant of each sample was assayed in triplicate. Total soluble protein was measured by the Bradford method (1976) using bovine serum albumin as a standard.

Amylase activity was assayed by the method of Bernfeld (1955), using soluble starch (Merck) as the substrate and reacting it with 3,5-dinitrosalicylic acid. Total protease activity was assayed according to a method modified from that of Anson (1938), using casein (Merck) as the substrate and reacting it with Folin reagent. Lipase activity was determined using a method modified from Mongkolthanaruk and Dharmsthiti (2002) using ρ-nitrophenyl palmitate as the substrate. Enzyme activities were measured as the change in absorbance using a Shimadzu 160-UV spectrophotometer.

2.5. Measurement of survival and growth

At the end of each experiment, the percent survival was determined and 50 shrimp were sampled randomly from each tank or pond to determine wet weight and total length. In Experiment III, carapace length also was measured; feed conversion ratio (FCR) and specific growth rate (SGR) were determined using the following equations:

$$FCR = \frac{\text{dry weight of ingested food}}{\text{wet weight of produced shrimp}}$$

$$SGR = \frac{(\ln W_t - \ln W_0) \times 100}{t}$$

where *t* is the culture period in days, ln*W*₀ is the natural logarithm of the weight of the shrimp at beginning of the experiment, and ln*W*_{*t*} is the natural logarithm of the weight of the shrimp at day *t*. (*W*₀ and *W*_{*t*} are in g.)

2.6. Statistical analysis

A randomized block design was used in each of the three experiments. Normality of data was tested using

Table 1

Total bacterial count and *Bacillus* count in water and in digestive tracts of *F. indicus* reared with and without *Bacillus* probiotic added to water or to food

Stage	Treatment	Water			Digestive tract			
		Total counts (10 ⁶ CFU/ml)	<i>Bacillus</i> counts (10 ⁴ CFU/ml)	Ratio (%) (<i>Bacillus</i> /total)	Total counts (10 ⁶ CFU/sample) [‡]	<i>Bacillus</i> counts (10 ⁴ CFU/sample) [‡]	Ratio (%) (<i>Bacillus</i> /total)	
Experiment I	Z ₃	C	0.11 ± 0.01 ^a	0 ^b	0 ^d	0.0063 ± 0.0003 ^f	0 ^h	0 ^j
		P _w	0.11 ± 0.01 ^a	9.6 ± 0.2 ^c	87.2 ± 2.1 ^e	0.0086 ± 0.0002 ^g	0.80 ± 0.02 ⁱ	93 ± 4.0 ^k
Experiment II	PL ₁₋₂	C	1.0 ± 0.2 ^a	0 ^c	0 ^f	1.0 ± 0.1 ⁱ	0 ^k	0 ^m
		P _w	1.6 ± 0.1 ^b	3.6 ± 0.1 ^d	2.3 ± 0.4 ^g	1.4 ± 0.1 ^{ij}	2.0 ± 0.2 ^l	1.4 ± 0.2 ⁿ
	PL ₁₄	P _a	1.5 ± 0.1 ^b	2.7 ± 0.2 ^c	1.8 ± 0.3 ^h	1.6 ± 0.1 ^j	3.6 ± 0.1 ^l	2.3 ± 0.2 ^p
		C	2.6 ± 0.1 ^a	0 ^b	0 ^d	2.5 ± 0.1 ^f	0 ^g	0 ^j
	PL ₁₄	P _w	2.4 ± 0.2 ^a	190 ± 10 ^e	79.1 ± 3.0 ^c	2.6 ± 0.1 ^f	160 ± 20 ^h	61.5 ± 2.5 ^k
		P _a	2.6 ± 0.2 ^a	160 ± 10 ^e	61.5 ± 4.7 ^c	2.5 ± 0.1 ^f	220 ± 10 ⁱ	88.0 ± 2.0 ^l
Experiment III	PL ₈₆	C	41 ± 1 ^a	0 ^b	0 ^d	14.3 ± 0.3 ^f	0 ^g	0 ^j
		P	38 ± 0 ^a	5.8 ± 0.0 ^c	0.15 ± 0.05 ^e	13.3 ± 0.8 ^f	0.91 ± 0.01 ^h	0.07 ± 0.01 ^k
	PL ₁₂₀	PP	40 ± 1 ^a	5.9 ± 0.1 ^c	0.15 ± 0.04 ^e	14.0 ± 0.6 ^f	3.7 ± 0.1 ⁱ	0.30 ± 0.09 ^l
		C	42 ± 0 ^a	0 ^b	0 ^d	19.0 ± 0.5 ^f	0 ^g	0 ^j
	PL ₁₂₀	P	42 ± 1 ^a	9.7 ± 0.1 ^c	0.23 ± 0.05 ^e	19.3 ± 0.3 ^f	2.1 ± 0.1 ^h	0.11 ± 0.01 ^k
		PP	41 ± 0 ^a	9.8 ± 0.0 ^c	0.24 ± 0.05 ^e	19.0 ± 0.6 ^f	2.8 ± 0.1 ⁱ	0.15 ± 0.01 ^l

Mean ± S.E. indicated (*N*=5 for water samples; *N*=20 for digestive tract samples). Means with the same superscript (within the same ontogenetic stage within a given experiment) are not significantly different (*P*>0.05). Z₃—zoea stage 3; PL—postlarval stages; C—controls (no probiotic provided); P_w—probiotic added to water; P_a—probiotic added in enriched *Artemia* nauplii provided as food; P—shrimp received the probiotic only in farming stages (PL₃₀ to PL₁₂₀); PP—shrimp received the probiotic both in the hatchery stages (N₁₋₂ to PL₃₀) and in the farming stages.

[‡] in Experiments I and II, “CFU/larva” or “CFU/postlarva”; in Experiment III, “CFU/g of digestive tract”.

the Anderson–Darling test (MINITAB 13.31 software). All data means were compared using Duncan's multiple range test (SAS software). A significance level of $P < 0.05$ was used for all tests. Data are reported as means \pm standard errors.

3. Results

3.1. Bacterial study

In all treatments, *Bacillus* bacteria in the probiotic successfully colonized both the culture water and the digestive tract of the shrimp (Table 1). The mean *Bacillus* count in probiotic-enriched *Artemia* was $1.6 \pm 0.2 \times 10^3$ CFU/nauplius. For either water or digestive tract samples, total bacterial counts in probiotic treatments were not significantly different from total bacterial counts in controls. However, the flora in probiotic treatments was significantly different from the flora in controls. *Bacillus* bacteria were not detected in the water samples or digestive tract samples in the controls from any of the experiments, but *Bacillus* bacteria became artificially dominant (87.2–93.0% of total bacteria for treatment P_w) in both the water and the digestive tracts of Z₃ larvae in Experiment I. Although both the water and digestive tracts were only poorly colonized in stage PL_{1–2} of Experiment II (the ratio of *Bacillus* to total bacteria was only slightly elevated: 1.4–2.3% *Bacillus* for treatments P_w and P_a), *Bacillus* bacteria also become artificially dominant (61.5–88.0% of total bacteria) by stage PL₁₄ in both treatments. Even though increases in the proportion of *Bacillus* bacteria were significant in treatments compared to controls, colonization of both water and digestive tracts by *Bacillus* bacteria was extremely low in all treatments in the earthen pond trials (Experiment III), where *Bacillus* bacteria represented only 0.07–0.30% of total bacteria in treatments P and PP.

3.2. Enzyme activity

The specific enzyme activities of amylase and lipase were significantly higher ($P < 0.05$) in shrimp from all probiotic treatments than in control shrimp for all ontogenetic stages examined (Figs. 1, 2 and 3). In early larval stages (Z₃), specific protease

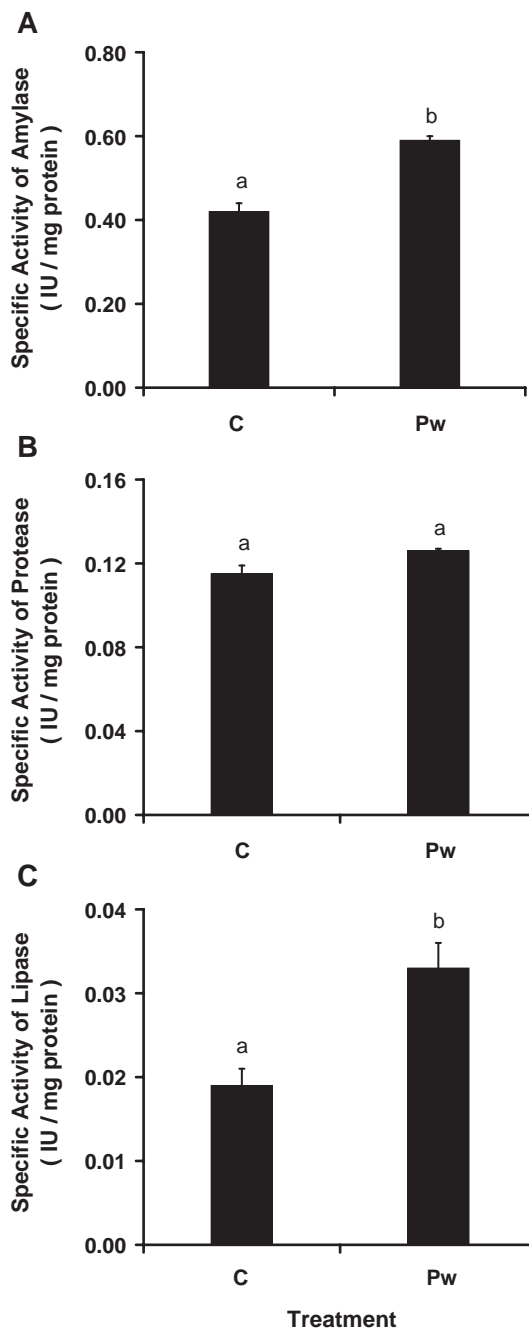


Fig. 1. Specific activities of enzymes in digestive tract of *F. indicus* at zoea stage 3 (Z₃), reared with and without *Bacillus* probiotic added to water starting at nauplius stages 1 and 2 (N_{1–2}) in Experiment I. Mean \pm S.E. indicated; $N = 20$. Means with the same super-script are not significantly different ($P > 0.05$). C—controls (no probiotic added); P_w—probiotic added to water.

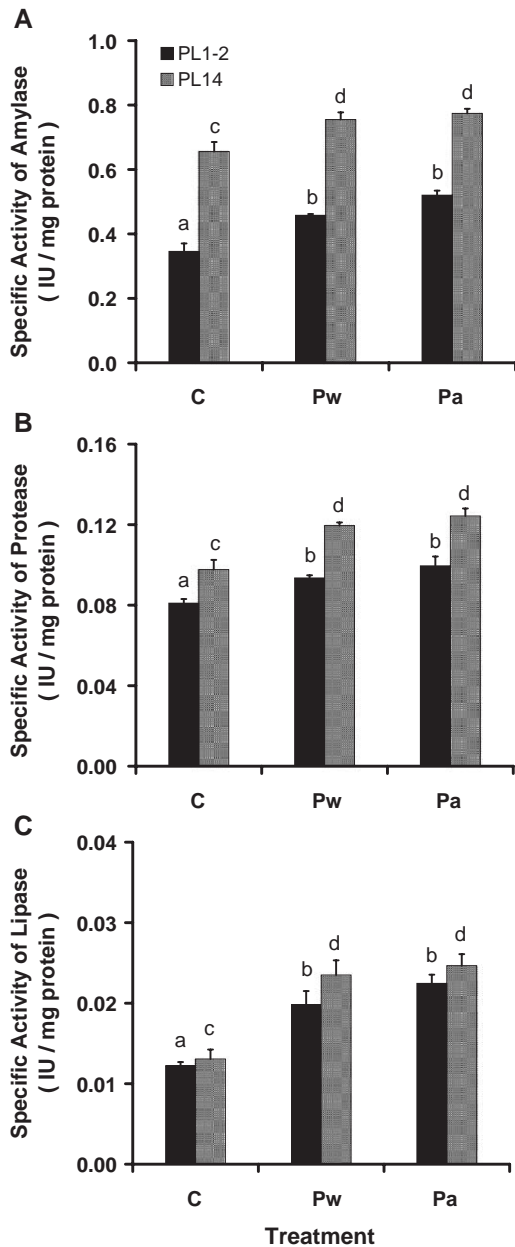


Fig. 2. Specific activities of enzymes in digestive tract of *F. indicus* reared with and without *Bacillus* probiotic added to either water or food starting at mysis stage 1 (M_1) in Experiment II. Mean \pm S.E. indicated; $N=20$. Means within the same ontogenetic stage with the same superscript are not significantly different ($P>0.05$). C—controls (no probiotic added); P_w—probiotic added to water; P_a—probiotic added in enriched *Artemia* nauplii provided as food; PL₁₋₂—postlarvae 1–2 days after metamorphosis; PL₁₄—postlarvae 14 days after metamorphosis.

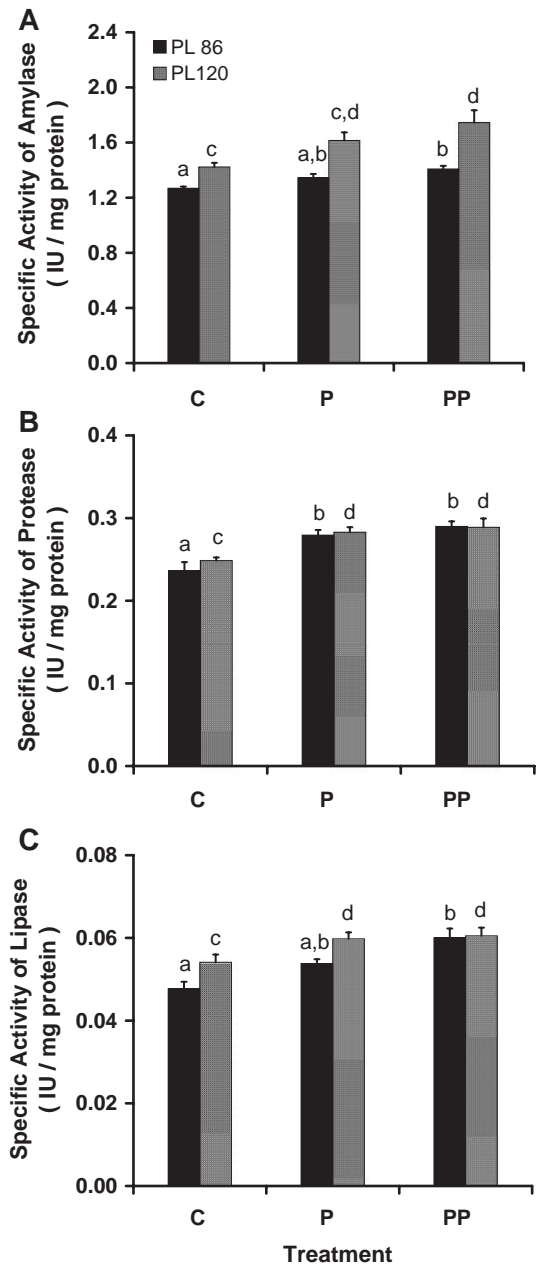


Fig. 3. Specific activities of enzymes in digestive tract of *F. indicus* reared with and without *Bacillus* probiotic added to water starting at either nauplius stage 1 and 2 (N_{1-2}) or PL₃₀ in Experiment III. Mean \pm S.E. indicated; $N=20$. Means within the same ontogenetic stage with the same superscript are not significantly different ($P>0.05$). PL—postlarval stages; C—controls (no probiotic provided); P—probiotic provided only in farming stages (PL₃₀ to PL₁₂₀); PP—probiotic provided both in hatchery stages (N_{1-2} to PL₃₀) and in the farming stages.

Table 2

Survival and growth parameters of *F. indicus* reared with and without *Bacillus* probiotic added to water or to food for N_{1–2} to Z₃ (Experiment I) and for M₁ to PL_{1–2} (Experiment II)

	Treatment	Survival (%)	Wet weight (mg)	Total length (mm)
Experiment I	C	66.3 ± 1.3 ^a	0.14 ± 0.01 ^c	2.0 ± 0.04 ^d
	P _w	78.1 ± 1.2 ^b	0.17 ± 0.01 ^c	2.1 ± 0.04 ^d
Experiment II	C	59.8 ± 0.9 ^a	15.83 ± 0.07 ^c	16.9 ± 0.09 ^e
	P _w	75.1 ± 3.2 ^b	19.02 ± 0.13 ^d	17.5 ± 0.15 ^{e,f}
	P _a	74.0 ± 2.0 ^b	19.32 ± 0.24 ^d	18.0 ± 0.20 ^f

Mean ± S.E. indicated. *N*=3 for % survival; *N*=150 for all other means. Means with the same superscript within the same experiment are not significantly different (*P*>0.05). C—controls (no probiotic provided); P_w—probiotic added to water; P_a—probiotic added in enriched *Artemia* nauplii provided as food.

activity in shrimp was not significantly different among probiotic treatments and controls. In contrast, in subsequent ontogenetic stages (PL_{1–2} through PL₁₂₀), specific protease activity was significantly higher in shrimp in all probiotic treatments than in control shrimp. Administration of the probiotic by feeding enriched *Artemia* to the shrimp consistently resulted in significant increases in specific activities of digestive enzymes in treatments (P_a) over those in controls; addition of the probiotic directly to the water also resulted in significantly higher specific activities of some enzymes in treatments (P_w) than in controls (for example, amylase and lipase in Experiments I and II) (Fig. 2). Furthermore, addition of the probiotic to the water in both hatchery and farming stages (treatment PP in Experiment III) resulted in a significant increase (*P*<0.05) in specific activities of all enzymes, as compared to controls, but there was no significant difference between these and treatments (P) that had received the probiotic only in the farming stage. Furthermore, specific enzyme activities in shrimp in treatment P were not always significantly (*P*<0.05) higher than in controls.

3.3. Survival and growth

Administration of the probiotic significantly increased survival in all treatments (generally by 10–15%) over controls, except in treatment P in Experiment III, where survival was not significantly different from that in controls (Tables 2 and 3). There was no significant difference (*P*>0.05) in either total length or carapace length between controls and those treatments in which probiotic was added to the water. In contrast, in those treatments where shrimp were fed probiotic-enriched *Artemia* (P_a), total length was significantly greater (*P*<0.05) than in controls (Tables 2 and 3). Where probiotic was administered to early larval stages (Experiment I), wet weight was not significantly different (*P*>0.05) between treatment and control shrimp, but when probiotic was administered to later larval and early postlarval stages (Experiment II), wet weight was significantly greater in treatments than in controls; there was no difference in wet weight between P_w and P_a (Table 2). When probiotic was administered both in hatchery and farming stages (treatment PP), final production, FCR, and SGR were significantly higher (*P*<0.05) than in controls,

Table 3

Growth and survival parameters of *F. indicus* reared with and without *Bacillus* probiotic added to water for PL₃₀ to PL₁₂₀ in earthen ponds (Experiment III)

Treatment	Survival (%)	Wet weight (g)	Total length (cm)	Carapace length (cm)	Final Production (kg/100 m ²)	FCR	SGR (%)
C	71.5 ± 4.25 ^a	12.26 ± 0.37 ^c	12.17 ± 0.18 ^c	2.58 ± 0.03 ^f	19.06 ± 3.91 ^g	1.98 ± 0.06 ^l	5.85 ± 0.03 ^k
P	82.25 ± 6.08 ^{a,b}	13.22 ± 0.48 ^{c,d}	12.36 ± 0.10 ^c	2.63 ± 0.04 ^f	22.06 ± 3.90 ^{g,h}	1.87 ± 0.06 ^{l,j}	5.93 ± 0.04 ^{k,l}
PP	88.53 ± 1.71 ^b	14.31 ± 0.28 ^d	13.47 ± 0.68 ^c	2.46 ± 0.02 ^f	25.56 ± 1.00 ^h	1.73 ± 0.07 ^j	6.02 ± 0.02 ^l

Mean ± S.E. indicated. *N*=3 for % survival; *N*=150 for all other means. Means with the same superscript are not significantly different (*P*>0.05). C—control (no probiotic provided); P—received the probiotic only in farming stage (PL₃₀ to PL₁₂₀); PP—received the probiotic both in hatchery stages (N_{1–2} to PL₃₀) and in the farming stages.

but when probiotic was administered to the farming stage only (treatment P), there were no significant differences between treatment and controls in each of these parameters (Table 3).

4. Discussion

The presence of the *Bacillus* probiotic significantly improved shrimp survival in most treatments. Because, administration of the probiotic significantly changed the proportion of *Bacillus* bacteria in the gut flora, the increased survival by shrimp may be due to exclusion of other bacteria (especially harmful bacteria) by the probiont, particularly in the larval and early postlarval stages where the *Bacillus* bacteria were dominant. In *P. monodon*, *Bacillus*, used as a probiotic, was able to colonize both the culture water and the shrimp digestive tract; the *Bacillus* also was able to replace *Vibrio* spp. in the gut of the shrimp, thereby increasing shrimp survival (Rengpipat et al., 1998a). *Bacillus* bacteria are able to out-compete other bacteria for nutrients and space and can exclude other bacteria through the production of antibiotics (Moriarty, 1998; Verschuere et al., 2000). However, the extent to which competitive exclusion may occur in the earthen pond treatments is Experiment III is not clear, given the very low colonization rates by *Bacillus* bacteria. Many different antibiotic compounds are produced naturally by a range of *Bacillus* species, and it appears that other bacteria would be unlikely to have resistance genes to all of the antibiotics produced by the *Bacillus* probionts, especially if they had not been exposed to the *Bacillus* previously (Moriarty, 1998). *Bacillus* administration also has been shown to increase shrimp survival by enhancing resistance to pathogens by activating both cellular and humoral immune defenses in shrimp (Rengpipat et al., 2000). *Bacillus* surface antigens or their metabolites act as immunogens for shrimp by stimulating phagocytic activity of granulocytes (Itami et al., 1998).

Administration of the *Bacillus* bacteria to shrimp resulted in an increase in the specific activity of lipase, protease and amylase in the shrimp's digestive tract. Because gram-positive bacteria, particularly members of the genus *Bacillus*, do secrete a wide range of exoenzymes (Moriarty, 1996, 1998), we cannot distinguish between activity due to enzyme synthesized

by the shrimp and activity due to enzyme synthesized by the bacteria. However, the low proportion of *Bacillus* bacteria in the gut of shrimp in Experiment III suggests that the exogenous enzymes produced by the probiont would contribute at most a small amount to the total enzyme activity of the gut. Perhaps, instead, the presence of the probiont may in some way stimulate endogenous enzymes produced by the shrimp. The observed increases in specific activities of digestive enzymes in probiotic treatments may have led to enhanced digestion and increased absorption of food, which in turn contributed to the improved survival and growth in *F. indicus*, including improved feed conversion ratio (FCR) and specific growth rate (SGR). In contrast, Shariff et al. (2001) and McIntosh et al. (2000) found that treatment of *P. monodon* and *Litopenaeus vannamei* with a commercial *Bacillus* probiotic did not significantly increase ($P > 0.05$) either survival or growth.

For shrimp receiving probiotic in both the hatchery and the farming stages (treatment PP in Experiment III), all of the growth parameters except total length and carapace length were significantly higher in treatments than in controls, while there was no significant difference in these parameters between controls and those shrimp that had received probiotic only during the farming stage (treatment P). There also were significantly more *Bacillus* bacteria in the digestive tracts of shrimp that had received probiotic in both stages (PP) than in shrimp that had received probiotic during only the farming stages (P), although the colonization rate was very low in both treatments. The correlation of higher bacterial counts with higher digestive enzyme activity and improved survival and growth parameters in treatment PP over controls strongly suggests that adding the probiotic during the hatchery stages and continuing its administration throughout the farming stages is necessary to maximize survival and growth in the shrimp. Administration of the probiotic only to the farming stages (PL₃₀–PL₁₂₀, treatment P in Experiment III) did not significantly improve survival or growth above that observed in controls, whereas adding probiont to both nursery and farming stages (treatment PP) did significantly increase survival and growth.

This investigation also examined the effectiveness of *Artemia* enrichment with the *Bacillus* bacteria. Because *Artemia* is a non-selective filter feeder, uptake

of bacteria by *Artemia* nauplii is strongly dependent upon the type of bacteria used, duration of exposure, and state (live or dead) of the bacteria (Gomez-Gil et al., 1998). We found that feeding probiotic to *Artemia*, followed by feeding these enriched *Artemia* to the shrimp, was an effective means by which to deliver the probiotic to the shrimp. However, administering probiotic in this manner did not significantly improve survival or growth, and therefore, the additional labor and expense involved in this manner of administration would not be cost effective.

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References

- Anson, M.L., 1938. The estimation of pepsin, trypsin, papain and cathepsin with hemoglobin. *J. Gen. Physiol.* 22, 79–89.
- Bernfeld, P., 1955. Amylase. In: Colowick, S.P., Kaplan, N.O. (Eds.), *Methods in Enzymology*. Academic Press, New York, pp. 149–158.
- Bradford, M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. *Anal. Biochem.* 72, 248–254.
- Brito, R., Rosas, C., Chimal, M.E., Gaxiola, G., 2001. Effect of different diets on growth and digestive enzyme activity in *Litopenaeus vannamei* (Boone, 1931) early post-larvae. *Aquac. Res.* 32, 257–266.
- Fuller, R., 1989. Probiotics in man and animal. *J. Appl. Bacteriol.* 66, 365–378.
- Gatesoupe, F.J., 1999. The use of probiotics in aquaculture. *Aquaculture* 180, 147–165.
- Gomez-Gil, B., Herrera-Vega, M.A., Abreu-Grobois, F.A., Roque, A., 1998. Bioencapsulation of two different *Vibrio* species in nauplii of the brine shrimp (*Artemia franciscana*). *Appl. Environ. Microbiol.* 64, 2318–2322.
- Itami, T., Asano, M., Tokushige, K., Kubono, K., Nakagawa, A., Takeno, N., Nishimura, H., Maeda, M., Kondo, M., Takahashi, Y., 1998. Enhancement of disease resistance of Kuruma shrimp, *Penaeus japonicus*, after oral administration of peptidoglycan derived from *Bifidobacterium thermophilum*. *Aquaculture* 164, 277–288.
- Lovett, D.L., Felder, D.L., 1990. Ontogenic change in digestive enzyme activity of larval and postlarval white shrimp *Penaeus setiferus* (Crustacea, Decapoda, Penaeidae). *Biol. Bull.* 178, 144–159 (Woods Hole).
- McIntosh, D., Samocha, T.M., Jones, E.R., Lawrence, A.L., McKee, D.A., Horowitz, S., Horowitz, A., 2000. The effect of a commercial bacterial supplement on the high-density culturing of *Litopenaeus vannamei* with a low-protein diet in an outdoor tank system and no water exchange. *Aquac. Eng.* 21, 215–227.
- Mongkolthananurak, W., Dharmstithi, S., 2002. Biodegradation of lipid-rich wastewater by a mixed bacterial consortium. *Int. Biodeterior. Biodegrad.* 50, 101–105.
- Moriarty, D.J.W., 1996. Microbial biotechnology: a key ingredient for sustainable aquaculture. *Infofish Int.* 4, 29–33.
- Moriarty, D.J.W., 1998. Control of luminous *Vibrio* species in penaeid aquaculture ponds. *Aquaculture* 164, 351–358.
- Rengpipat, S., Phianphak, W., Piyatiratitivorakul, S., Menasveta, P., 1998a. Effects of a probiotic bacterium on black tiger shrimp *Penaeus monodon* survival and growth. *Aquaculture* 167, 301–313.
- Rengpipat, S., Rukpratanporn, S., Piyatiratitivorakul, S., Menasveta, P., 1998b. Probiotics in aquaculture: a case study of probiotics for larvae of the black tiger shrimp (*Penaeus monodon*). In: Flegel, T.W. (Ed.), *Advances in Shrimp Biotechnology*. National Center for Genetic Engineering and Biotechnology, Bangkok.
- Rengpipat, S., Rukpratanporn, S., Piyatiratitivorakul, S., Menasveta, P., 2000. Immunity enhancement on black tiger shrimp (*Penaeus monodon*) by a probiotic bacterium (*Bacillus S11*). *Aquaculture* 191, 271–288.
- Ribeiro, F.A.L.T., Jones, D.A., 2000. Growth and ontogenetic change in activities of digestive enzymes in *Fenneropenaeus indicus* postlarvae. *Aquac. Nutr.* 6, 53–64.
- Shariff, M., Yusoff, F.M., Devaraja, T.N., Srinivasa Rao, S.P., 2001. The effectiveness of a commercial microbial product in poorly prepared tiger shrimp, *Penaeus monodon* (Fabricius), ponds. *Aquac. Res.* 32, 181–187.
- Thompson, F.L., Abreu, P.C., Cavalli, R., 1999. The use of microorganisms as food source for *Penaeus paulensis* larvae. *Aquaculture* 174, 139–153.
- Verschuere, L., Rombaut, G., Sorgeloos, P., Verstraete, W., 2000. Probiotic bacteria as biological control agents in aquaculture. *Microbiol. Mol. Biol. Rev.* 64, 655–671.
- Ziaei-Nejad, S., 2004. The effects of *Bacillus* spp. bacteria as a probiotic on growth, survival and digestive enzyme activity of Indian white shrimp, *Fenneropenaeus indicus*, larvae and post-larvae. MSc thesis. Tehran University. 100 pp.