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Effect of *Bacillus subtilis* on *Aeromonas hydrophila* infection resistance in juvenile freshwater prawn, *Macrobrachium rosenbergii* (de Man)

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Abstract This experiment was carried out to investigate the potential probiotics properties of *Bacillus subtilis* in protecting of juvenile freshwater prawn, *Macrobrachium rosenbergii* against *Aeromonas hydrophila* infection. A *B. subtilis* bacterium isolated from gut of juvenile prawns was added to the prawns feed at 10^8 cells g^{-1} feed. There were significant differences ($P < 0.05$) between *B. subtilis*-treated and control groups in growth and survival enhancement of juvenile prawns after 60 days of feeding trial. Sixty days after *B. subtilis* feeding trial, the prawns were challenged by bath exposure to *A. hydrophila* (10^7 cells mL^{-1}) for 28 days. Four weeks after challenge, there was significant difference in the survival of prawns between *B. subtilis*-treated groups (88.33 %) and control groups (20.81 %; $P < 0.05$). In addition, the control groups had an unhealthy external appearance, while the treated groups, appeared healthy and normal. From this, it was concluded that the selected *B. subtilis* may be a promising probiotics for protection of prawns from *A. hydrophila* infection.

Keywords *M. rosenbergii* · Probiotics · Challenge test · *A. hydrophila* · *B. subtilis*

Introduction

The giant freshwater prawn, *M. rosenbergii* (de Man), has commanded significant attention in tropical freshwater aquaculture research and development. The characteristics that make the culture of this species attractive include breeding easily in captivity, high hatching rate, high quality meat and in many countries, high market value. However, the relatively low survival of larval and juvenile stages often prevents the development of full economic

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potential of this crustacean (New et al. 2010). The majority of the freshwater prawn mortality can be attributed to the involvement of pathogenic bacteria (Hoa et al. 2000; Scan 2003; Keysami et al. 2007). The *Aeromonas* spp., *Enterococcus* spp. and *Vibrio* spp. are among the most important bacterial pathogens of cultured prawns responsible for a number of diseases (Keysami et al. 2007). Probiotics have been considered a possible solution to reduce these diseases problems. During the recent years, several trials were conducted with micro-organisms known to be probiotics to improve the culture of food species and to improve human health and welfare (Rengpipat et al. 1998; Verschuere et al. 2000). Probiotics are live micro-organisms that when consumed in adequate amount confer a health benefit for the host (FAO/WHO 2001). Appropriate probiotics applications were shown to improve intestinal microbial balance, thus leading to improved feed utilization (FAO 2007; Keysami et al. 2012) and reduced pathogenic problems in the gastrointestinal tract (Scan 2003). In the aquaculture industry, several probiotics species have been used, including *Lactobacillus* sp. (Abraham et al. 2007), *Saccharomyces* sp. (Rumsey et al. 2007), *Bacillus* sp. (Balcazar et al. 2004; Meunpol et al. 2003; Keysami et al. 2007) and mixed cultures (Sotomayure and Balcazar 2003; Meunpol et al. 2003; Salinas et al. 2005; Aly et al. 2008). The trials demonstrated the growth promotion of fish and crustaceans compared to control groups. Those results were most promising and gave confidence that further improvements in probiotics applications were possible (Irianto and Austin 2002). Austin et al. (1995) found that the probiont *Vibrio alginolyticus* applied to salmon (*Salmo salar*) could reduce diseases caused by *A. salmonicida*, *V. anguillarum* and *V. ordalii*. However, probiotics application in aquaculture was extensive, especially for freshwater prawn culture (Venkat et al. 2004).

It is apparent that the majority of probiotics for nutritional effect comprise lactic acid bacteria, notably *Lactobacillus* sp., *Bifidobacterium* sp. and *Streptococcus* sp. (Irianto and Austin 2002), because lactic acid bacteria formed a common and important component of the normal micro-biota in the gastrointestinal tracts of healthy fish and mammals (Ringo and Gatesoupe 1998; Abraham et al. 2008). There are no reports of lactic acid bacteria in *M. rosenbergii* as a common component of micro-biota (Cai et al. 1999; Venkat et al. 2004; Keysami et al. 2005), but *Bacillus* sp. is known to form a major component of the normal micro-biota in the *M. rosenbergii* gastrointestinal tracts (Colorni 1985; Anderson et al. 1989; Keysami et al. 2005). Therefore, the objective of this research was to evaluate the effect of *B. subtilis* on *A. hydrophila* infection resistance in juvenile freshwater prawn, *M. rosenbergii* (de Man).

Materials and methods

Preparation of experimental prawns and design

A feeding trial experiment and a challenge test were conducted from 25 June to 25 September in 2010. A total of 240 juvenile prawns (*M. rosenbergii*) with no external signs of infection and no behavioral abnormalities were obtained from a hatchery at Ahvaz, Iran. The forty juvenile prawns of size ranging from 0.39 to 0.46 g were introduced into each of 6 glass aquaria of 60 L capacity ($60 \times 30 \times 30$ cm^3). The mean weights of prawns in 6 aquaria were not significantly different ($P > 0.05$). The aquaria were filled with 360 L tap water with characteristics included pH = 7.17; electrical conductivity (Ec) = 0.16 mmhos; Water temperature (T) = 27.21 °C; dissolved oxygen (DO) = 7.16 mg L^{-1} and ammonia–nitrogen = 0.00 mg L^{-1} according to New et al. (2010).

Upon initiation of the experiment, juvenile prawns were acclimatized in 6 aquaria for 1 week and were fed with a commercial pellet feed (Havorash, Bushehr, IRI) containing crude protein (34.93 ± 0.15), crude lipid (6.48 ± 0.37), crude fiber (8.26 ± 0.14) and ash (17.02 ± 0.29) three times daily. The basic ingredients as per the manufacturer of the feed included fish meal, shrimp shell meal, fish oil, squid liver powder, wheat flour, soya meal, lecithin, vitamin and mineral premixes.

Feed trays were used for prawns feeding. The uneaten feed fraction was removed and accounted after 30 min of feeding. Daily feeding was 5–8 % of total prawns body weight. Feed ration was altered every week based on the estimated feed consumption rate of prawns. The bottom of each aquarium was thoroughly cleaned once a week, at the same time approximately 50–100 % of the water was exchanged. Throughout the experimental rearing period water was well aerated. Photoperiod was maintained at 12 h Light with artificial fluorescent lighting. Weights of 10 randomly collected prawns from each tank were determined every 2 weeks. Prawn survival was also determined by counting dead prawns in each tank daily. These data were used to estimate survival, wet weight gain (WG), feed conversion ratio (FCR), specific growth rate (SGR) and feed intake (FI) according to keysami et al. (2012):

$$\begin{aligned}
\text{WG (g)} &= \text{Final weight (g)} - \text{initial weight (g)} \\
\text{WG (\%)} &= \text{WG (g)/initial weight (g)} \times 100 \\
\text{FCR} &= \text{Total feed given (g)/WG (g)} \\
\text{SGR (\%)} &= (\ln \text{FPW} - \ln \text{IPW})/t \times 100 \\
\text{FI (g)} &= \text{Total feed consumed (g)/}[(\text{INP} + \text{FNP})/2] \\
\text{Survival (\%)} &= (\text{FNP}/\text{INP}) \times 100
\end{aligned}$$

where: t = time (days); ln = natural logarithmic; INP = initial number of prawns; FNP = final number of prawns; IPW = initial prawn body wet weight; FPW = final prawn body wet weight.

Diet preparation

A *B. subtilis* bacterium isolated from juvenile *M. rosenbergii* gut as described in keysami et al. (2012) were used as probiotics strain. This strain was identified by using biochemical tests according to Bergey et al. (1984), and Biolog Diagnostic System kit and Microstation (Biolog, Inc., CA, USA).

The experimental diets were designated as *B. subtilis*-treated feed (PF) and control feed (CF). The *B. subtilis*-treated diet was prepared according to Keysami et al. (2012) by soaking commercially available *M. rosenbergii* pellet feed (Havorash, Bushehr, IRI) with normal saline (NSS; Nacl 8.5 g L^{-1}) containing the *B. subtilis* fresh cells. Bacterium was grown for 48–72 h at 30 °C in Trypton Soy broth (Difco, CO, USA) and harvested by centrifugation 4,000*g* for 15 min. The cells were re-suspended in original volume of saline to 5×10^{13} cells mL^{-1}. This suspension was then applied to unsoaked diet to achieve a counted viable dose approximately equivalent to 5×10^{8} cells g^{-1} of feed after absorbing whole bacteria suspension during 1 h. This level of *B. subtilis* (10^{8} cells g^{-1}) was chosen based on earlier works of researchers (Meunpol et al. 2003; Keysami et al. 2012). The control diet also was prepared by soaking the same basis pellet feed with normal saline without the *B. subtilis* fresh cells. The resulting pellets were oven-dried at 32 °C for 1 h

and then dried pellets (200 g portions of the feed) were packed in sealed plastic bags and used for experiment or stored at 4 °C.

Pathogen challenge test

A pathogenic bacterium, *A. hydrophila* , obtained from faculty of veterinary, University of Tehran, was used in this challenge experiment by immersion assay after 60 days feeding trial. *A. hydrophila* had been cultured and maintained using TSB broth and agar. Prawns in all replicates were immersed in a suspension of *A. hydrophila* at 10^7 cells mL^{-1} according to Austin et al. (1995). This was followed by a re-immersion of 10^7 cells mL^{-1} after 7 days (Rengpipat et al. 1998). Water and prawns were collected and checked every 2 days from each tank for microbial examination and prawn survival during the immersing test. The challenge experiment was carried out for a period of 28 days in duplicates and the prawns feeding was continued with experimental diets.

Prawns in each treatment groups were dissected by sterile surgical scissors and examined morphologically and microscopically at day 28 of the challenge test. Prawn survival was also determined for each replicate. *A. hydrophila* isolated from prawns' gut were purified and identified using biochemical tests, and were compared with the original *A. hydrophila* cultures. Confirmation of the identity of *A. hydrophila* was done by using Biolog identification kit and software.

Water quality parameters

Water quality was monitored daily. Temperature and pH were measured using an YSI (Yellow Spring Inc.), pH and temperature meter (60–10 FT), respectively. Dissolved oxygen was estimated by an YSI, DO and temperature meter model 57 (USA) and ammonia–nitrogen was estimated by an ammonia meter, HANNA instrument (HI 93715 m; Taiwan), respectively.

Bacteriological study

Prawns feces (200–300 mg) and one live prawn were collected from each tank, once every 2 weeks for bacterial determination. Prawns were dissected using sterilized surgical scissors and their gut was removed for bacterial enumeration and evaluation. Bacterial enumeration was made using serial dilution in NSS (NaCl 8.5 g L^{-1}) at 10 folds, followed by spread plating on nutrient agar and tryptic soy agar (Difco, CO, USA). After 24 h of incubation at 32 °C, plate colonies were counted and recorded. Bacterial strains were re-examined using selected morphological and biochemical tests.

Statistical analysis

Data on growth parameters, prawns survival, water quality, bacteria counts between replicates and treatments were analyzed according to Zar (1984) by using two independent samples *t* test in SPSS, release16, software (SPSS, Inc., USA). The level of significance was accepted at ($P < 0.05$).

Results

Physico-chemical parameters of rearing water

Physico-chemical parameters like water temperature (°C), dissolved oxygen (mg L^{-1}), pH and ammonia–nitrogen were in ranges of 27.23–29.73 °C, 5.5–7.06 mg L^{-1}, 7.8–8.07 and 0.011–0.038 mg L^{-1}, respectively. Values were stable and were within the recommended optimum range for *M. rosenbergii* culture (New et al. 2010). There were no significant differences in water physico-chemical variables between control and treated tanks ($P > 0.05$).

Bacteriological study

Bacteria from the gut and feces of juvenile prawns after feeding the *B. subtilis* for 60 days were found to be *Bacillus* with characteristics similar to the *B. subtilis* that were used as feed additive.

Bacterial counts in prawns' gut and prawns feces during 60 days of feeding with diet prepared with *B. subtilis* were presented in Tables 1 and 2. Tables 1 and 2 showed the same pattern of bacterial counts in prawns' gut and feces with different values. After 60 days of culture, gut total bacteria count from each culture tanks was $6.3 \pm 2.6 \times 10^7$ cells mL^{-1}, regardless of treatments. *Bacillus* spp. concentration increased to $2.5 \pm 2.02 \times 10^6$ cells mL^{-1} in treated tanks through the 60 days, but no increment found in the control tanks ($2 \pm 0.06 \times 10^2$ cells mL^{-1}). Gram-negative bacteria concentrations were $1.1 \pm 0.33 \times 10^2$ cells mL^{-1} in all treated tanks and $4 \pm 1.02 \times 10^5$ cells mL^{-1} in the control tanks through the 60 days.

Gut bacteria of juvenile prawns in control and treated groups after challenge for 28 days were found to be *Aeromonas* sp. and *Bacillus* sp., respectively. Bacterial counts in prawns' gut and in rearing tanks water during 28 days of challenge were presented in Figs. 1 and 2. During the challenge test, *A. hydrophila* concentrations in both rearing water and prawns' gut of the control groups increased to 10^7 cells mL^{-1}, while only 10^2 cells mL^{-1} of *Bacillus* spp. were detected. Conversely, with treated groups, the probiont *B. subtilis* was the dominant bacteria (10^8–10^9 cells g^{-1}), with lower concentrations of *A. hydrophila* in

Table 1 Mean log total viable cells bacteria (cells g^{-1}), *Bacillus* and gram-negative bacteria counts in prawns' gut during 10 days of 60 days feeding diets

Days	Total bacteria count		*Bacillus* count		Gram-negative bacteria count	
	CF	PF	CF	PF	CF	PF
0	2.6^a	2.6^a	1^a	1^a	2.6^a	2.6^a
2	3.9^a	5.7^b	1	2.8^b	3.4^a	2.01^b
4	4.7^a	5.9^b	1	4.6^b	4.3^a	2.02^b
6	5.3^a	6.3^b	1	6.1^b	4.9^a	2.03^b
8	7.8^a	7.8^a	1^a	7.4^b	5.9^a	2.05^b
10	2.6^a	2.6^a	1^a	1^a	2.6^a	2.6^a

These diets were designated as *B. subtilis*-treated feed (PF) and non-*B. subtilis*-treated feed (CF), respectively

All values are means of three replicates per treatment

Values containing same superscript in a row for each parameter do not vary significantly ($P > 0.05$)

Table 2 Mean log total viable cells bacteria (cells g^{-1}), *Bacillus* and gram-negative bacteria counts in prawns feces during 60 days of feeding diets

Days	Total bacteria count		*Bacillus* count		Gram-negative bacteria count	
	CF	PF	CF	PF	CF	PF
0	2.5^{a}	2.5^{a}	1^{a}	1^{a}	2.4^{a}	2.4^{a}
2	3.9^{a}	3.9^{a}	1^{a}	2.3	2.6^{a}	2.4^{a}
4	4.3^{a}	4.3^{a}	1^{a}	3^{b}	3.4^{a}	2.5^{b}
6	5.3^{a}	5.3^{a}	1^{a}	3.5^{b}	3.9^{a}	2.5^{b}
8	5.8^{a}	5.9^{b}	1^{a}	5.2^{b}	5.3^{a}	2.6^{b}
10	2.5^{a}	2.5^{a}	1^{a}	1^{a}	2.4^{a}	2.4^{a}

These diets were designated as *B. subtilis*-treated feed (PF) and non-*B. subtilis*-treated feed (CF), respectively

All values are means of three replicates per treatment

Values containing same superscript in a row for each parameter do not vary significantly ($P > 0.05$)

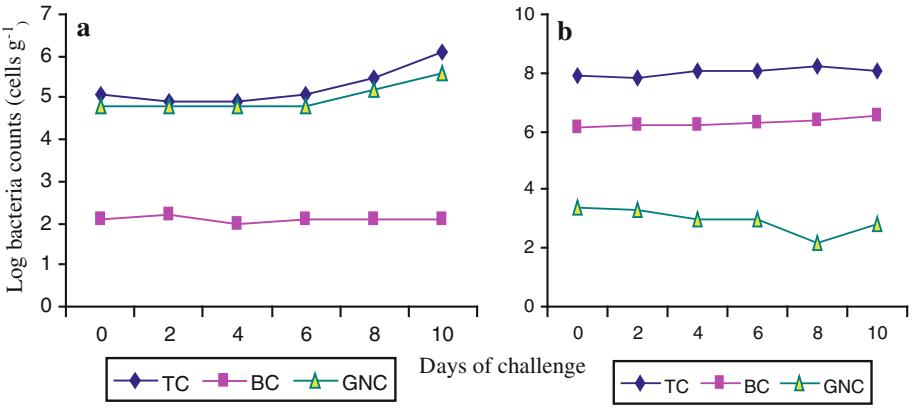

Fig. 1 Log total bacteria (TC), *Bacillus* (BC) and gram-negative (GNC) bacteria counts (cells g^{-1}) in prawns' gut during 28 days of challenge in control (**a**) and *B. subtilis*-treated tanks (**b**)

water (10^2 cells mL^{-1}) and in the gut (2×10^2 cells g^{-1}). The main gram-negative bacteria found in all challenge tanks were identified as *A. hydrophila.*

Growth parameters

There were significant differences ($P < 0.05$) in weight gain, specific growth rate, feed intake and FCR between the treated and control groups (Table 3). Treated groups mean wet weight gain (1.49 ± 0.03) was significantly ($P < 0.05$) higher than control groups (0.615 ± 0.03). Prawn survival after 60 days was significantly higher ($P < 0.05$) in the treated groups (84.23 ± 1.72) than the control groups (78.22 ± 2.24 %; Table 3).

After 28 days of challenge with *A. hydrophila*, survival of *B. subtilis*-treated groups (88.33 %) was significantly higher ($P < 0.05$) than the control groups (20.81 %; Table 4). Moreover, no external appearance, disease signs and behavioral abnormalities was observed in the treated groups. They looked normal in size, color and appearance, whereas

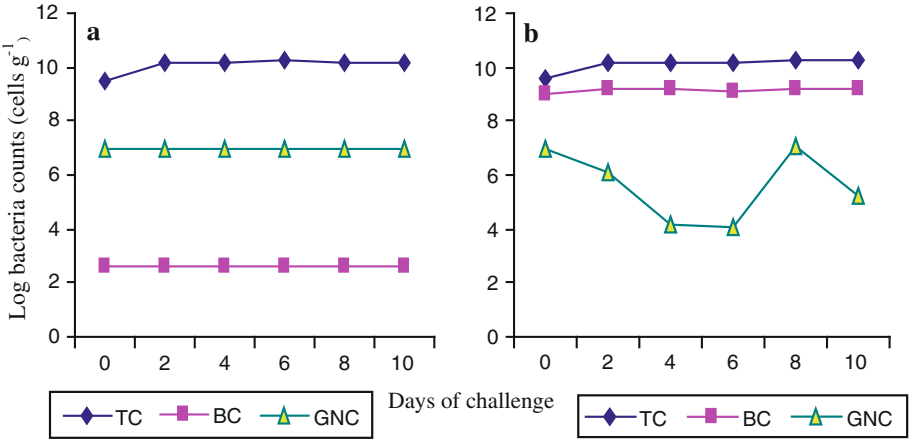

Fig. 2 Log total bacteria (TC), *Bacillus* (BC) and gram-negative bacteria (GNC) counts (cells mL^{-1}) in prawn rearing tank water during 28 days of challenge in control tank (**a**) and *B. subtilis*-treated tank (**b**)

Table 3 Growth-related performance of *M. rosenbergii* during 60 days of feeding diets

Growth-related performance	CF	PF
Initial mean weight (g)	0.45 ± 0.05^{a}	0.43 ± 0.01^{a}
Final mean weight (g)	1.06 ± 0.03^{a}	1.92 ± 0.02^{b}
Weight gain (g)	0.62 ± 0.03^{a}	1.49 ± 0.03^{b}
Weight gain (%)	137.56 ± 6.34^{a}	347.88 ± 17.53^{b}
Feed intake (g)	2.14 ± 0.02^{a}	3.12 ± 0.06^{b}
FCR	3.48 ± 0.12^{a}	2.10 ± 0.03^{b}
SGR	1.44 ± 0.05^{a}	2.51 ± 0.09^{b}
Survival (%)	78.22 ± 2.24^{a}	84.23 ± 1.72^{b}

These diets were designated as *B. subtilis*-treated feed (PF) and non-*B. subtilis*-treated feed (CF), respectively

All values are means of three replicates per treatment (mean ± SD)

Values containing same superscript in a row do not vary significantly ($P > 0.05$)

infected prawns in the control groups exhibited black spot, necrosis of uropods, lost periopods and pale hepatopancrease.

Discussion

In this study, it was well demonstrated that the use of *B. subtilis* as probiotics could efficiently increase the growth and survival of juvenile prawns. These may be attributed to the possibility of higher feed intake and SGR related to treated groups (Keysami et al. 2007). The recent reports on the use of *Bacillus* spp. (Balcazar et al. 2004; Salinas et al. 2005; Keysami et al. 2012) also demonstrated the beneficial effects of stimulating the growth improvements and gut immune system in the fish and prawn (Abraham et al. 2008). After 60 days of feeding, total bacterial count in water from each culture tank was

Table 4 Survival of juveniles *M. rosenbergii* in *B. subtilis*-treated (PF) and control groups (CF) challenged with *A. hydrophila* during 28 days

Time (days)	CF	PF
0	100.00 ± 0.00^{a}	100.00 ± 0.00^{a}
7	63.31 ± 2.93^{a}	100.00 ± 0.00^{b}
14	49.22 ± 1.43^{a}	98.32 ± 1.43^{b}
21	30.81 ± 3.81^{a}	96.73 ± 1.72^{b}
28	20.81 ± 1.43^{a}	88.33 ± 1.53^{b}

These diets were designated as *B. subtilis*-treated feed (PF) and non-*B. subtilis*-treated feed (CF), respectively

All values are means of two replicates per treatment (mean ± SD)

Values containing same superscript in a column do not vary significantly ($P > 0.05$)

10^{10} cells mL^{-1}, regardless of treatment. The same result was reported by Keysami et al. (2012). The *B. subtilis* concentration increased to 10^{8}–10^{10} cells mL^{-1} in all treated tanks through the first 60 days, but was not found in the control tanks. *Bacillus* spp. concentrations were 2×10^{2} cells mL^{-1} in the control tanks. *Bacillus* spp. presence may have been due to non-competitive, saprophytic growth. Nearly similar findings were reported using *Bacillus* sp. as probiotics in the *Litopenaeus vannamei* feed (Balcazar et al. 2004; Gullian et al. 2004). It is possible that this phenomenon operates by substitution of opportunist pathogens that reduced growth (Balcazar et al. 2004).

These results indicate that the *B. subtilis* replaced other bacterial species in the culture water. Total bacterial flora, both gram-positive and gram-negative rods, were found in prawns' gut in all treated and control tanks on days 7–8, were in a range of 10^{9}–10^{10} cells g^{-1}; similar to results reported by Keysami et al. (2012). The main flora in prawns' gut of the control groups were gram-negative (10^{7} cells g^{-1}) and a few *Bacillus* spp. (10^{2} cells g^{-1}), while those in all the treated groups were mostly *Bacillus* sp. (10^{8}–10^{9} cells g^{-1}) and a few gram-negative bacteria (10^{2} cells g^{-1}). It appears that, gram-negative bacteria in treated prawns' gut were replaced by *B. subtilis*. This same pattern of bacterial replacement occurred in prawns' feces of treated groups, where gram-negatives were replaced by *Bacillus* sp. Alternatively, *B. subtilis* prevented potential pathogens from colonizing the gut by production of antimicrobial compounds or by out competing them for nutrients or mucosal space (Gullian and Rodriguez 2002; Vaseeharan and Ramasamy 2003).

Treatment with *B. subtilis* appeared to enhance growth and survival of *M. rosenbergii*. Gram-negative bacteria in treated prawns' gut were replaced by *Bacillus* sp. This same pattern of bacterial replacement occurred in rearing tank water and prawns' gut of treated groups in challenge test, where *A. hydrophila* was replaced by *Bacillus* sp in rearing tanks water and prawns' gut in *B. subtilis*-treated tanks. Gram-negative bacteria are the dominant opportunistic bacterial flora in the digestive tracts of prawns. These bacteria are transients and may change rapidly with the intrusion of probiotics bacteria coming from water or feed (Abraham et al. 2008).

Although *B. subtilis*-treated groups were also immersed in *A. hydrophila*, they resisted from both external and internal infection of *A. hydrophila*. More importantly, these results are an indirect indication that the *B. subtilis* viably colonize prawns' gut and proliferate in a manner that benefited the host (Renault et al. 2007). The *B. subtilis* colonization of prawns' gut apparently acted as an interferer or competitor against *A. hydrophila* infection.

The *B. subtilis* may produce some antimicrobial substances, or some unknown by-products negatively affected *A. hydrophila* (Balcazar et al. 2004).

Result of bacterial counts in gut and feces showed that there were significant differences between bacterial counts of prawns in control and treated groups. Gram-negative bacteria counts in prawns of treated groups were significantly lower than control groups. The *B. subtilis* has been shown to produce a wide variety of antibacterial and antifungal compounds in culture media (Chythanya and Karunasagar 2002; Gullian et al. 2004). It produces novel antibiotics such as difficidin and oxydifficidin that have activity against a wide spectrum of aerobic and anaerobic bacteria (Sugita et al. 2002; Gullian et al. 2004). It is expected that gram-negative bacteria would be replaced with probiont strain (Vine et al. 2004). It is assumed that strains showing a dominant colonization of the intestinal mucus of fish and culture water are good candidates to competitively exclude pathogens from the adhesion sites of the gut wall (Wang et al. 2000). However, the use of *B. subtilis* in prawns feed can reduce juvenile *M. rosenbergii* mortalities during culture. Although the results of this study could be the basis for future studies, our findings should be confirmed in earthen pond trials before they are applied commercially.

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