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Probiotic activity of *Bacillus subtilis* in juvenile freshwater prawn, *Macrobrachium rosenbergii* (de Man) at different methods of administration to the feed

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Abstract This experiment was carried out to investigate the potential probiotic properties of *Bacillus subtilis* and suitable methods of administration to the commercial feed in juvenile *Macrobrachium rosenbergii* (de Man) from 25 August to 25 November 2008. Putative *Bacillus subtilis* bacterium isolated from juvenile *M. rosenbergii* intestine was added to commercial prawn feed as a probiotic. Five types of diets were prepared by mixing *B. subtilis* at level of 108 cells g^{-1} with commercial feed using different methods consisting mixing, soaking, spraying and bathing. After 60 days, the prawns fed diets at soaking method treated group, showed a higher mean weight gain (2.09) or 328.84% increase in growth over control. There were significant differences ($P < 0.05$) in weight gain, feed intake and FCR among soaking method and other treated and control groups. There was significant difference ($P < 0.05$) in survival among treated and control groups, but no significant difference ($P > 0.05$) in water quality and biochemical composition among treated and control groups. Clearly, *B. subtilis*-treated diets appeared to enhance growth and survival of juveniles *M. rosenbergii*. It was concluded that the tested strain may be a promising probiotic for *M. rosenbergii* under soaking method of administration to the prawn commercial feed.

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Introduction

There is rapid increase in the world aquaculture activities (FAO 2007). Particularly in the last few years, the freshwater prawn *M. rosenbergii* has been recognized as a species with great aquaculture potential (New 2005). At present, the intensification of production, decrease in water quality, increase in stress and decrease in food quality can suppress the growth of freshwater prawn production (Keysami et al. 2007). The opportunity exists for feeds to be developed to attain better feed conversion ratios. The use of probiotic in aquafeeds has received considerable attention in recent years. The rationale of their use in aquaculture is to improve feed intake, feed efficiency and survival and to minimize feed wastage and water pollution (Verschuere et al. 2000).

A negative aspect of the success of aquaculture has been increased intensification leading to outbreaks of diseases, encompassing an ever increasing range of pathogens (Scan 2003). To combat these diseases, widespread use of broad-spectrum antibiotics has led to drug resistance problems (Scan 2003). Recently, in order to rectify this situation, attention has been focused on the use of probiotics. Probiotics are organisms and substances that contribute to intestinal microbial balance. Most probiotics are supplied as live supplements in food and therefore must have the ability to survive passage through the intestinal tract (Verschuere et al. 2000). Probiotics are live microorganisms, which, when consumed in adequate amounts, confer a health benefit for the host (FAO/WHO 2001).

In the aquaculture industry, several probiotic species have been used, including *Lactobacillus* spp. (Abraham et al. 2007), *Saccharomyces* sp. (Rumsey et al. 2007), *Bacillus* spp. (Balcazar et al. 2004; Meunpol et al. 2003; Keysami et al. 2007) and mixed cultures (Sotomayure and Balcazar 2003; Salinas et al. 2005; Aly et al. 2008). 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 major component of the normal microflora in the gastrointestinal tracts of healthy fish and mammals (Ringo and Gatesoupe 1998). There are no reports of lactic acid bacteria in *M. rosenbergii* as a common component of microflora (Venkat et al. 2004; Keysami et al. 2005), but *Bacillus* sp. *is known to* form a major component of the normal microflora in the *M. rosenbergii* (Colorni 1985; Anderson et al. 1989; Keysami et al. 2005). Therefore, we propose that *Bacillus* sp. be used as putative probiotic in *M. rosenbergii*, in a manner similar to lactic acid bacteria in mammals and fish. The effect of candidate probiotics should be tested in vivo as well. When the probiotic effect is supposed to be nutritional, the candidate probiotic could be added to the culture of the aquatic species and their effect on growth and survival parameters could be assessed. Although *B. subtilis* in aquafeed was tested for their efficacy as has already been demonstrated in animal production systems, methods of its application in the feed were not tested. Unlike animal production practices, use of probiotics in aquafeeds may have limitations due to water exposure. Therefore, the development of suitable method for incorporation of probiotics in the aquafeeds is needed (Venkat et al. 2004). This study was designed to study different methods of *B. subtilis* application in the commercial feed of juvenile *M. rosenbergii*.

Materials and methods

Experimental animals and design

A total of 600 healthy juvenile *M. rosenbergii* prawns were obtained from University Putra Malaysia (UPM) hatchery and individually weighed and placed in fifteen 60-l aquaria each measuring 60 × 30 × 30 cm (40 animals per an aquarium). Prawns were weighed to the nearest 0.01 g, using an electronic balance (AND, EK 1200, Japan), after being blot-dried on a paper towel. At the beginning of the experiment, the mean weight (±SD) of the prawns in the 3 aquaria of the control group was 0.45 ± 0.03 g. The mean weight of the prawns in the other aquaria which were to receive the probiotic treatment was 0.43 ± 0.05 g. These mean weights were not significantly different ($P > 0.05$). Juvenile prawns were acclimatized in 15 aquaria for 1 week and were fed commercial pellet feed as like as control feed (Cargile, BHD, Malaysia) three times daily.

This experiment was conducted as a completely randomized design with five treatments from 25 August to 25 November 2008. Each treatment had three replicates of 40 prawns each. Prawns were fed, with diets, prepared under different methods of *B. subtilis* administration to the prawn commercial feed, at level of (10^8 cells g^{-1}), three times daily at 6.00 h (40%), 12.00 h (20%) and 18.00 h (40%). This level of *B. subtilis* was chosen based on earlier works of researchers (Nikoskelainen et al. 2001; Meunpol et al. 2003; Keysami et al. 2007). Feed trays were used for prawn feeding. The uneaten feed fraction was removed and accounted at 30 min after feeding. Daily feeding rate was 5–8% of prawn body weight throughout the experimental rearing period. Daily feeding ration was modified each week based on the estimated feed consumption rate of prawns. The bottom of each aquarium was thoroughly cleaned once each week, at the same time approximately 50–100% of the water was exchanged. Throughout the experimental rearing period, water was well aerated. Weights of 10 randomly collected prawns from each tank were determined every 2 weeks. Prawn survival was also determined by removing dead prawns in each aquarium daily.

Diet preparation

A *B. subtilis* from 12 groups of putative bacterial flora isolated from *M. rosenbergii* juvenile gut that possessed the greatest antibacterial activity against *Aeromonas hydrophila* and *Vibrio parahaemolyticus* using an agar diffusion technique (Sugita et al. 2002) was selected for this investigation. *Aeromonas hydrophila* and *Vibrio parahaemolyticus* obtained from Faculty of Veterinary at UPM. *B. subtilis* strain was gram positive, rod shape, catalase positive and spore former. These strains were isolated and identified by using biochemical tests according to Bergey et al. 1984, and Biolog Diagnostic System kit and Microstation (Biolog, Inc., California, USA). *B. subtilis* was cultured in tryptic soy broth (Difco, Colorado, USA) and stocked on tryptic soy agar (Difco, Colorado, USA). Cultures were incubated at 32°C in 2-l flasks for 24 h, and then, *B. subtilis* cells were centrifuged (15 min, 4,000×*g*). Centrifuged cells washed in sterile normal saline solution (NSS; Nacl 8.5 g l^{-1}) three times and stored at −20°C until they were used. Fresh cells at 24 h, at a concentration of 10^{13} (cells ml^{-1}) of cells wet weight, were harvested and maintained at 4°C for a maximum of 2 weeks or at −20°C for longer periods of stocking. *B. subtilis* cells were brought to room temperature from −20°C and were used as fresh cells 3.0 × 10^{13} cells ml^{-1}. The normal saline solution (NSS; Nacl 8.5 g l^{-1}) was added to the bacterial fresh cells to achieve 10^{10} (cells ml^{-1}) of saline. This solution was added to

commercial pellet feed to give an initial number of 10^8 (cells g^{-1}) of feed. Four types of diets were prepared using the same base feed by altering the method of administrating of *B. subtilis* to the feed at the level of 10^8 (cells g^{-1}) of feed, as described below, except the control. Probiotic-treated feed *by mixing* (PF1), probiotic-treated feed by *soaking* (PF2), probiotic-treated feed by *spraying* (PF3), probiotic-treated feed by *bathing* (PF4), respectively. Control diet (non-probiotic-treated feed) was designated as CF.

Mixing method (PF1)

This method was carried out according to Nikoskelainen et al. (2001) and Meunpol et al. (2003). The commercial prawn pellet feed was blended to a small particle size in a laboratory Mill, Thoms-Wiley Model 4, USA. Fresh cell suspension was added to the grounded feed. The dough was kneaded thoroughly and repeated for proper mixing. The dough was then extruded in a noodle-making machine (La parmigiana, Model D45 LE, Italy) through 1 mm diameter. The resulting pellets were oven-dried at 35°C for 1–2 h, and then, they were made into crumbles of about 4 mm in diameter. The crumbles were stored in sealed plastic bags and kept at 4°C for 2 weeks or −20°C until they were used as PF1. Final concentration of *Bacillus* in treatment feeds was determined to be 3.3×10^8 cells g^{-1} feed.

Soaking method (PF2)

Soaking method described by Robertson et al. (2000) was modified to prepare these probiotic feed (PF2). Probiotic diets were prepared by soaking commercially available *M. rosenbergii* pelleted feed with normal saline containing the fresh cells of *B. subtilis*. *B. subtilis* bacterium was grown for 48–72 h at 35°C in trypton soya broth and harvested by centrifugation at 4,000×*g* for 15 min. The cells were resuspended in original volume of saline to 5×10^{13} cells ml^{-1}. This suspension was then applied to un-soaked diets by mixing in a mixer for 15 min and to achieve a viable dose equivalent to 5.1×10^8 cells g^{-1} of feed after absorbing whole bacteria suspension during 1 h. The resulting pellets were oven-dried at 32°C for 1–2 h, and then, dried pellets (200-g portions of the feed) were packed in sealed plastic bags and stored at 4°C for 2 weeks or −20°C until they were used.

Spraying methods (PF3)

The commercial prawn pellet feeds were sprayed by pumping the suspensions of *B. subtilis* with normal saline (10^{10} cells ml^{-1}) through a syringe onto the dry feed pellets that were placed in a plastic tray on the rectangle surface of shaker according to Gildberg and Mikkelsen (1998). After spraying, the mixture was oven-dried at 35°C for 1–2 h. Finally, 200-g portions of the Bacillus supplemented feed (4.7×10^8 cells g^{-1}) were packed in sealed plastic bags and stored at 4°C for 2 weeks or −20°C until used as PF3. The moisture content was reduced to about 5%, which was slightly less than the initial moisture content of the commercial feed before spraying.

Bathing method (PF4)

B. subtilis was cultured in tryptic soy broth (Difco) and stocked on tryptic soy agar. Culture conditions were at 35°C in 2-l flasks for 24–72 h, after which *B. subtilis* cells were centrifuged (15 min, 4,000×*g*) and washed in sterile normal saline solution three times,

and then, fresh cells aged 24 h at 10^{13} cells g^{-1} of cells wet weight were harvested and maintained at 4°C until 2 week or −20°C to long time prior to use. *Bacillus* cells were brought to room temperature and were used as fresh cells. A suspension of *B. subtilis* with normal saline (10^{10} cells ml^{-1}) was directly added to rearing water to reach 4.6×10^8 cells ml^{-1} of culture water using the sterile beaker according to Gullian et al. 2004.

Growth analysis

The growth parameters were calculated by using the following formulae according to (Felix and Sudharsan 2004; Venkat et al. 2004):

$$\text{Weight gain (g)} = \text{Final weight (g)} - \text{Initial weight (g)}$$

$$\text{Weight gain (\%)} = \frac{\text{Final weight (g)} - \text{Initial weight (g)}}{\text{Initial weight (g)}} \times 100$$

$$\text{Food conversion ratio (FCR)} = \frac{\text{Total feed given (g)}}{\text{Wet weight gain (g)}}$$

$$\text{Mean feed intake (g)} = \frac{\text{Total feed consumed (g)}}{(\text{Initial number of prawns} + \text{Final number of prawns})/2}$$

$$\text{Survival (\%)} = \frac{\text{Number of prawns survived at the end of the experiment}}{\text{Number of prawns stocked at the start of the experiment}} \times 100$$

Proximate analysis

The moisture, crude protein, lipid, ash and crude fiber contents in the control feed and treated diets and prawn body tissue were analyzed according to the standard procedures of Association of Official Analytical Chemist (1990). Moisture was determined by oven drying at 105°C for 24 h and protein by Kjeldahl method after acid digestion. Crude lipid was determined in Soxhlet apparatus by extracting the residue with 40–60°C petroleum ether for 8 h. Crude fiber was determined as loss on ignition of dried lipid-free residues after digestion with 1.25% H_2SO_4 and 1.25% NaOH, and ash was determined by ignition at 550°C in a muffle furnace to constant weight. The nitrogen-free extract (NFE) was calculated by using the following formula:

$$\text{NFE} = 100 - (\text{crude protein} + \text{crude lipid} + \text{ash} + \text{crude fiber} + \text{moisture}).$$

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 meter; Taiwan), respectively.

Bacteriological study

Prawn feces (200–300 mg) and one live prawn were collected from each tank, once every 2 weeks for bacterial determination starting from the first day of the feeding trials. Prawns

were dissected using sterilized surgical scissor, and the gut and intestine were removed for bacterial enumeration and evaluation. Bacterial determinations were 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). After 24–48 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, biochemical composition, prawns survival, water quality and bacteria count between replicates and treatments were analyzed by using one-way analysis of variance, and significance of differences between treatments were assessed by Duncan multiple range test (Sokal and Rohlf 1995). The level of significance was accepted at ($P < 0.05$). All statistical analyses were performed using SPSS, release12, software (SPSS, Inc., USA).

Results

Physico-chemical parameters of rearing water

Mean physico-chemical parameters like water temperature (°C), dissolved oxygen (mg l^{-1}), pH and ammonia–nitrogen were recorded in ranges from 27.27 to 29.9°C, 5.2–7.8 (mg l^{-1}), 8–8.3 and 0.011–0.018 (mg l^{-1}), respectively (Table 1). Values were stable and were within the recommended optimum range for *M. rosenbergii* culture (New et al. 2010). There were no significant differences in physico-chemical variables of control and treatments tanks ($P > 0.05$).

Biochemical analysis of experimental diets and tissues

Chemical composition of the experimental diets and tissues was reported in Tables 2 and 3. The feed crude protein content ranged from 34.46 to 35.05%, and crude lipid content varied from 6.41 to 6.68%. Tissue crude protein was recorded in PF1 (68.2) and PF4 (67.17%), respectively. Tissue crude lipid content was recorded within range of 3.27–3.6%. There was no significant difference in biochemical composition variables of control and treated groups ($P > 0.05$) during 60 days feeding trial.

Growth parameters

The growth parameters recorded in this experiment were presented in Table 4. Among the four different methods used of *B. subtilis* application (mixing, soaking, spraying and bathing), soaking performed the best in all the growth-related parameters. The prawns fed diet PF2 (soaking method) showed a higher ($P < 0.05$) mean weight gain (2.09 ± 0.06) and 328.84% increase in weight gain% over control. There were significant differences ($P < 0.05$) for weight gain, specific growth rate, feed intake and FCR between PF2 and other treated and control groups. There were no significant differences ($P > 0.05$) for weight gain, specific growth rate, feed intake and FCR between the PF1 and PF3. Significant variation ($P < 0.05$) was observed in survival of the prawns between control and

Table 1 Water quality of *M. rosenbergii* culture water by using diets prepared with different methods of *B. subtilis* administration to the feed (mean ± SD)

Treatments	CF	PF1	PF2	PF3	PF4
T (°C)					
Am					
Mean	27.23[a]	27.07[a]	27.07[a]	27.27[a]	27.13[a]
SD (±)	0.4	0.06	0.21	0.25	0.15
Pm					
Mean	29.73[a]	29.9[a]	29.53[a]	29.27[a]	29.73[a]
SD (±)	0.4	0.3	0.5	0.29	0.23
DO (mg l^{-1})					
Am					
Mean	5.7[a]	5.6[a]	5.5[a]	5.6[a]	5.2[a]
SD (±)	0.4	0.47	0.55	0.25	0.1
Pm					
Mean	7.06[a]	7[a]	7.03[a]	7.03[a]	6.7[a]
SD (±)	0.15	0.42	0.21	0.47	0.47
pH					
Am					
Mean	7.9[a]	7.8[a]	7.8[a]	8.03[a]	8.01[a]
SD (±)	0.12	0.22	0.22	0.45	0.1
Pm					
Mean	8.07[a]	8.03[a]	8[a]	8.3[a]	8.3[a]
SD (±)	0.05	0.32	0.35	0.55	0.26
Ammonia (mg l^{-1})					
Am					
Mean	0.011[a]	0.012[a]	0.03[a]	0.02[a]	0.02[a]
SD (±)	0.004	0.003	0.02	0.01	0.01
Pm					
Mean	0.014[a]	0.014[a]	0.013[a]	0.013[a]	0.018[a]
SD (±)	0.004	0.001	0.002	0.002	0.008

These diets were designated as probiotic-treated feed by mixing (PF1), probiotic-treated feed by soaking (PF2), probiotic-treated feed by spraying (PF3), probiotic-treated feed by bathing (PF4) and non-probiotic-treated feed (CF), respectively

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

treated groups. The mean feed intake of the prawns fed diets prepared by soaking method was higher (3.21 ± 0 g) than the other diets. The prawn fed the control diet recorded poor feed intake. Best FCR value (2.10 ± 0.03) was obtained in prawn fed diet prepared by soaking method. The prawn fed control diet showed poor FCR value (Table 4). Generally, the growth and FCR performance were higher in all prawn fed the *B. subtilis-treated* diets than prawn fed control diet.

Mean prawn weights of 4 *B. subtilis*-treated groups (1.11–2.09 g) were significantly greater ($P < 0.05$) than control. Prawn survival after 60 days was significantly greater ($P < 0.05$) in the treated groups (84.2–85.3%) compared with the control group (78.2%; Table 5). Clearly, *B. subtilis under soaking application appeared* to enhance growth and

Table 2 Proximate composition of diets prepared with different methods of *B. subtilis* administration to the feed (% DM basis, mean ± SD)

Diets	CF	PF1	PF2	PF3	PF4
Crude protein	34.93[a]	35.05[a]	34.46[a]	35.03[a]	34.95[a]
SD (±)	0.15	0.05	1.17	0.06	0.15
Crude lipid	6.48[a]	6.41[a]	6.54[a]	6.47[a]	6.68[a]
SD (±)	0.37	0.43	0.37	0.25	0.24
CF	8.26[a]	8.21[a]	8.43[a]	8.15[a]	8.35[a]
SD (±)	0.14	0.1	0.08	0.05	0.14
Ash	17.02[a]	17.05[a]	17.15[a]	17.02[a]	17.08[a]
SD (±)	0.29	0.32	0.18	0.45	0.17
NFE	33.27[a]	33.28[a]	33.41[a]	33.33[a]	32.94[a]
SD (±)	0.78	0.67	1.37	0.35	0.45

These diets were designated as probiotic-treated feed by mixing (PF1), probiotic-treated feed by soaking (PF2), probiotic-treated feed by spraying (PF3), probiotic-treated feed by bathing (PF4) and non-probiotic-treated feed (CF), respectively

Values are means of three replicates per treatment

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

Table 3 Proximate composition of prawns whole body tissues (% DM basis, mean ± SD) by using diets prepared with different methods of *B. subtilis* administration to the feed

Diets	Initial	CF	PF1	PF2	PF3	PF4
Crude protein	63.9[a]	68.2[a]	66.27[a]	65.53[a]	65.73[a]	67.17[a]
SD (±)	1.35	2.1	1.07	0.59	0.32	0.35
Crude lipid	4.2[a]	3.6[a]	3.7[a]	3.57[a]	3.27[a]	3.51[a]
SD (±)	0.21	0.46	0.36	0.21	0.19	0.34
Ash	16.57[a]	15.97[a]	16.6[a]	17.03[a]	16.43[a]	16.06[a]
SD (±)	0.21	0.21	0.46	0.21	0.15	0.23
NFE	15.57[a]	12.23[a]	13.43[a]	13.87[a]	14.54[a]	13.26[a]
SD (±)	1.56	1.6	0.46	0.67	0.48	0.59
Moisture	75.6[a]	75.4[a]	75.25[a]	75.53[a]	75.57[a]	75.01[a]
SD (±)	0.56	0.37	0.26	0.49	0.49	0.37

These diets were designated as probiotic-treated feed by mixing (PF1), probiotic-treated feed by soaking (PF2), probiotic-treated feed by spraying (PF3), probiotic-treated feed by bathing (PF4) and non-probiotic-treated feed (CF), respectively

Values are means of three replicates per treatment

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

survival of *M. rosenbergii* significantly. *B. subtilis-treated diets* prepared with soaking showed greater efficacy ($P < 0.05$). Mixing and spraying methods gave similar results without any significant differences between these methods ($P > 0.05$).

Bacteriological study

Bacterial counts in prawn gut and feces during 60 days of feeding with diets prepared with different methods of administration for *B. subtilis* were presented in Tables 5 and 6. There

Table 4 Growth-related performance of *Macrobrachium rosenbergii* by using diets prepared with different methods of *B. subtilis* administration to the feed (mean ± SD)

Diets	CF	PF1	PF2	PF3	PF4
Initial mean weight (g)	0.45 ± 0.02^{a}	0.44 ± 0.01^{a}	0.43 ± 0.04^{a}	0.43 ± 0.01^{a}	0.44 ± 0.03^{a}
Final mean weight (g)	1.13 ± 0.06^{a}	1.899 ± 0.03^{c}	2.43 ± 0.045^{d}	1.86 ± 0.05^{c}	1.55 ± 0.069^{b}
Weight gain (g)	0.62 ± 0.08^{a}	1.46 ± 0.1^{b}	2.09 ± 0.06^{c}	1.45 ± 0.04^{b}	1.11 ± 0.08^{d}
Weight gain (%)	137.56 ± 6.34^{a}	331.76 ± 8.66^{b}	463.88 ± 17.53^{c}	336.46 ± 0.96^{b}	253.63 ± 8.86^{d}
Feed intake (g)	2.14 ± 0.02^{a}	3.1 ± 0.08^{c}	3.12 ± 0.06^{c}	3.21 ± 0^{c}	2.58 ± 0.03^{b}
FCR	3.48 ± 0.12^{a}	2.42 ± 0.01^{c}	2.10 ± 0.06^{d}	2.32 ± 0.06^{cb}	2.33 ± 0.09^{b}
Survival (%)	78.2 ± 2.2^{a}	85 ± 1.4^{b}	85 ± 2.9^{b}	84.3 ± 2.2^{b}	84.2 ± 1.7^{b}

These diets were designated as probiotic-treated feed by mixing (PF1), probiotic-treated feed by soaking (PF2), probiotic-treated feed by spraying (PF3), probiotic-treated feed by bathing (PF4) and non-probiotic-treated feed (CF), respectively

Values are means of three replicates per treatment

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

Table 5 Mean log total viable cells of bacteria, *Bacillus* and gram-negative bacteria count (cells g^{-1}) in prawn gut and intestine during 60 days of feeding diets prepared with different methods of *B. subtilis* administration to the feed

Diets	Time (week)	CF	PF1	PF2	PF3	PF4
Log total viable bacteria cells count	0	2.6	2.6	2.6	2.6	2.6
	2	3.9	5.7	5.7	5.7	4.3
	4	4.7	5.9	5.9	5.9	5.6
	6	5.3	6.3	6.3	6.3	5.9
	8	7.8^{a}	7.8^{a}	7.8^{a}	7.8^{a}	7.8^{a}
Log *Bacillus* sp. count	0	1	1	1	1	1
	2	1	2.8	2.8	2.8	2.6
	4	1	4.6	4.6	4.6	3.5
	6	1	6.1	6.1	6.1	4.9
	8	1^{a}	7.05^{b}	7.4^{c}	7.01^{b}	6.8^{c}
Log gram-negative bacteria count	0	2.6	2.6	2.6	2.6	2.6
	2	3.4	24	2.01	2.3	2.6
	4	4.3	2.3	2.01	2.3	2.9
	6	4.9	2.3	2.01	2.4	2.9
	8	5.9^{a}	2.3^{b}	2.01^{c}	2.4^{b}	3.2^{d}

These diets were designated as probiotic-treated feed by mixing (PF1), probiotic-treated feed by soaking (PF2), probiotic-treated feed by spraying (PF3), probiotic-treated feed by bathing (PF4) and non-probiotic-treated feed (CF), respectively

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

Table 6 Mean log total viable cells bacteria (cells g^{-1}), *Bacillus* and gram-negative bacteria count in prawn feces during 60 days of feeding diets prepared with different methods of *B. subtilis* administration to the feed

Diets	Time (week)	CF	PF1	PF2	PF3	PF4
Log total viable bacteria cells count	0	2.5	2.5	2.5	2.5	2.5
	2	3.9	3.9	3.9	3.9	3.6
	4	4.3	4.3	4.3	4.3	4.1
	6	5.3	5.3	5.3	5.3	4.9
	8	5.8^{a}	5.9^{a}	6^{a}	5.9^{a}	5.9^{a}
Log *Bacillus* sp. count	0	1	1	1	1	1
	2	1	2.3	2.3	2.4	2.3
	4	1	2.9	3	3.1	3
	6	1	3.3	3.5	3.6	3.2
	8	1^{a}	5.01^{b}	5.3^{c}	4.9^{b}	4.3^{d}
Log gram-negative bacteria count	0	2.4	2.4	2.4	2.4	2.4
	2	2.6	2.4	2.4	2.4	2.4
	4	3.4	2.6	2.5	2.4	2.6
	6	3.9	2.5	2.4	2.4	2.8
	8	5.3^{a}	2.5^{b}	2.2^{c}	2.6^{b}	3.1^{d}

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

These diets were designated as probiotic-treated feed by mixing (PF1), probiotic-treated feed by soaking (PF2), probiotic-treated feed by spraying (PF3), probiotic-treated feed by bathing (PF4) and non-probiotic-treated feed (CF), respectively

were significant differences between bacterial counts of prawns in control and treated groups ($P < 0.05$). There were significant differences between bacterial counts of prawns in PF2 and other treated groups ($P < 0.05$). Gut and feces bacteria of juvenile prawns after feeding *B. subtilis*-treated feed for 60 days were found to be *Bacillus* sp. based on similarities of their characteristics to those of the bacteria that used as feed additive. After 60 days of culture, *Bacillus* sp. count of gut and feces from soaking method (PF2) was significantly different from other treatments and control tanks (Tables 5 , 6). *Bacillus* sp. concentrations increased in PF1, PF2, PF3 and PF4 treatment tanks through the 60 days, but no increment in the control tanks was observed. *Bacillus* sp. concentrations were found to be $2 \pm 0.06 \times 10^{2}$ cells ml^{-1}in the control tanks. Gram-negative bacteria concentrations decreased in all treatment tanks through the 60 days, but its concentrations was $4 \pm 1.02 \times 10^{5}$ cells ml^{-1} in the control tanks.

Discussion

The probiotic activity of the *B. subtilis* was evident from the higher weight gain and better FCR recorded by prawns fed *B. subtilis*-treated diets over control feed. Results of 60 days *B. subtilis* feeding trial showed significant differences ($P < 0.05$) in weight gain, feed intake and FCR between the *B. subtilis*-treated and control groups. Mean weight gain of prawns in *B. subtilis*-treated group was significantly ($P < 0.05$) greater than those of prawns in control group. Prawns' survival after 60 days was also significantly greater

($P < 0.05$) in the *B. subtilis*-treated groups than in the control group. Nearly similar findings were reported by 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 the substitution of opportunist pathogens that reduced growth (Balcazar et al. 2004).

Probiotics may improve digestive activity by synthesis of vitamins and cofactors or improve enzymatic activity (Wang et al. 2000). These properties could be the cause of the weight increase, improving digestion or nutrient absorption. *B. subtilis* produces a variety of proteases and other enzymes that enable it to degrade a variety of natural substrates and contribute to nutrient cycling (Sugita et al. 2002; Gullian et al. 2004).

Alternatively, *B. subtilis* prevented potential pathogens from colonizing the gut by production of antimicrobial compounds or by outcompeting them for nutrients or mucosal space (Gullian and Rodriguez 2002).

In this study, the treatments PF1, PF2 and PF3 yielded prawn with significantly higher weight gain, FCR, feed intake and survival than PF4 and CF. Results of higher prawn growth rate from soaking, mixing and spraying probiotics administration methods were supported by several similar studies (Nikoskelainen et al. 2001; Meunpol et al. 2003). These studies reported higher growth rate for putative probiotics when were added to the host or its ambient environment in several ways including soaking, mixing, spraying, bathing and addition via live food.

The results of lower growth rates in bathing method as compared to soaking, spraying and mixing methods in this study could be due to lower *Bacillus* sp. count in prawn gut and feces. Hence, higher *Bacillus* sp. count is more effective (Gildberg et al. 1995). Bacteria have to be administered continuously to the feed, and a dose–effect relationship indicated that dosage of probiotics should be defined carefully (Nikoskelainen et al. 2001). When probiotics are administered in effective doses, they establish and colonize in the digestive tracts and increase the natural flora of the digestive tracts and the host's resistance to diseases (Gullian and Rodriguez 2002).

The growth rate of prawn was yielded higher in soaking method than mixing and spraying methods. The reason *is* higher losing of the bacteria from probiotic feed into the culture water in mixing and spraying methods (Nikoskelainen et al. 2001). The growth promoter effect is also conditioned to probiotic bacteria concentration in feed, which fed by prawns. Therefore, the results are subject to a stability of probiotic bacteria into the feed. Consequently, the probiotics used as growth stimulant can yield different results under different culture conditions methods of administration (Verschuere et al. 2000).

Result of bacterial count in gut and feces showed that there were significant differences between bacterial count of prawns in control and treated groups. Gram-negative bacteria count in prawns of control group was significantly higher than those of treated groups. *B. subtilis* has been shown to produce a wide variety of antibacterial and antifungal compounds in culture media (Chythanya and Karunasagar 2002; Sugita et al. 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 (Gullian et al. 2004). It is expected that gram-negative bacteria would 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. In conclusion, *B. subtilis* administration to the feed under soaking method was obviously demonstrated growth-enhancing effect in laboratory culture scale. The authors still believed that practical application in artificial culture scale may be limited and need to spend more time on this issue.

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