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Application of Probiotic Bacteria for Controlling Pathogenic Bacteria in Fairy Shrimp *Branchinella thailandensis* culture

Watchariya Purivirojkul[1,*]

[1] Department of Zoology, Faculty of Science, Kasetsart University, 10900, Chatuchak, Bangkok, Thailand.

* Corresponding Author: Tel.: 0.2562 5555; Fax: 0.2562 5555;
E-mail: fsciwyp@ku.ac.th

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Abstract

The use of probiotic bacteria for controlling black disease in fairy shrimp, *Branchinella thailandensis*, was studied. The bacterial antagonistic activities were tested with *Aeromonas hydrophila* WS1 which is pathogenic to fairy shrimp. The cross-streak method showed that after 48 hours, *Bacillus* W120 was the strongest inhibitor of *A. hydrophila* WS1. *Bacillus* W120 was characterized and confirmed as *B. vallismortis* by 16S rDNA sequence analysis. Competition, by using nutrient for growth, between *B. vallismortis* and *A. hydrophila* WS1 *in vitro* was studied. The amount of *A. hydrophila* WS1 co-cultured in nutrient broth with *B. vallismortis* W120 decreased by 84.14% after 48 hours. The toxicity of *B. vallismortis* W120 was tested by immersion challenge of fairy shrimp in bacteria suspension at concentrations of 1×10^4, 1×10^5 and 1×10^6 CFU/ml for 72 hours but no mortality was found in any treatment. Fairy shrimp were fed with feed containing *B. vallismortis* W120 at concentrations of 1×10^3, 1×10^4 and 1×10^5 CFU/ml for 7 days, and then challenged with *A. hydrophila* WS1. Fairy shrimp fed with *B. vallismortis* W120 at every concentration had a significantly higher survival rate than the control group, and the cumulative mortality rates among each *B. vallismortis* W120-treated group were not significantly different in every experiment period . These results suggest that *B. vallismortis* W120 can be applied as an effective probiotic in fairy shrimp culture to control the pathogenic bacteria, *A. hydrophila* WS1, by feeding shrimp this probiotic bacteria daily at a concentration of 1×10^3 CFU/ml .

Keywords: Black disease, fairy shrimp, probiotic, *Bacillus vallismortis.*

Introduction

In the last decade, fairy shrimp have begun to play a role in aquaculture because they have high hatching percentages and growth rates with low operational and maintenance costs, while most importantly, they are highly nutritious. The nutritional value of fairy shrimp is similar to *Artemia* but fairy shrimp have the potential to be used as a feed item for ornamental fish because their high carotenoid content makes them a prime food source for color enhancement in ornamental fish culture (Munuswamy, 2005) and they can be used as frozen food for aquarium fish (Boonmak *et al.*, 2007). Black disease in fairy shrimp, caused by *Aeromonas hydrophila*, occurs most frequently in the culture of fairy shrimp species such as *Branchipus schaefferi*, *Chirocephalus diaphanous*, *Streptocephalus torvicornis* and *Branchinella thailandensis* (Dierckens *et al.*, 1998; Muangsan *et al.*, 2006; Purivirojkul and Khidprasert, 2009). There have been several reports documenting the ability of probiotic bacteria, especially *Bacillus* spp., to control pathogenic bacteria in aquaculture, including the culturing of crustaceans such as *Penaeus monodon* (Rengpipat *et al.*, 1998; Meunpol *et al.*, 2003), *Litopenaeus vannamei* (Liu *et al.*, 2010) and *Homarus gammarus* (Daniels *et al.*, 2010). There are several mechanisms of probiotic action, including the production of inhibitory compounds, competition for chemicals or available energy, competition for adhesion sites, the enhancement of the immune response and improvement of water quality (Verschuere *et al.*, 2000a). A set of criteria has been proposed to select potential probiotic strains (Collins *et al.*, 1998). However, many reports have used *in vitro* production of inhibitory compounds toward known pathogens of a particular species as the first screen for selection of putative probiotic strains (Hjelm *et al.*, 2004). Many researchers have added probiotic organisms to live feed such as *Chaetoceros* spp., *Isochrysis galbana*, *Skeletonema costatum*, *Brachionus plicatilis* and

Artemia to improve production rates (Fukami *et al.*, 1992; Hagiwara *et al.*, 1994; Rombaut *et al.*, 1999; Kesarcodi-Watson *et al.*, 2008) and protect aquatic animals from pathogenic bacteria (Verschuere *et al.*, 2000b; Villamil *et al.*, 2003). In some cases, probiotic bacteria have been used in live feed such as *Artemia* or rotifers and fed to aquatic animals to enhance their growth rate and protect them from pathogenic bacteria (Gatesoupe, 1991; Rengpipat *et al.*, 1998). Therefore, probiotic organisms may act as prophylaxis against diseases caused by bacteria, especially *Aeromonas hydrophila*, in fairy shrimp and perhaps in aquatic animals that feed on fairy shrimp. Fairy shrimp could act as a carrier, transferring probiotic bacteria to aquatic animals and thus protecting them from the important pathogenic freshwater bacterium, *A. hydrophila.*

In this study, 13 strains of spore-forming bacteria that were previously shown to inhibit *A. hydrophila* were tested for antibacterial activity with *A. hydrophila* WS1, a strain isolated from infected fairy shrimp. Experiments were conducted *in vitro* and *in vivo* to determine the effective of probiotic bacteria for controlling black disease in cultures of fairy shrimp (*B. thailandensis*).

Materials and Methods

Experiment 1 : Pathogenic Bacterial Isolation and Identification

Fairy shrimp were collected from the Department of Fisheries, Faculty of Agriculture and Natural Resources, Rajamangala University of Technology Tawan-Ok, Chonburi province. A sample of 25 diseased fairy shrimp was investigated. The black parts were removed and identified following Bergey's manual of systematic bacteriology which are based on morphology, staining, oxygen requirements and biochemical tests (Holt *et al.*, 1994) and API20E identification systems (Biomérieux, France). Putative pathogenic bacteria were identified by 16S rDNA sequencing. Scanning electron microscopy (SEM) and the histopathology of diseased fairy shrimp were also used and the results studied. Isolates were stored at -80°C as suspensions in 15% glycerol.

Verification of Koch's Postulates

Pathogen-free fairy shrimp were used for challenge tests. The potential pathogenic bacterial strains were used for experimental infection in fairy shrimp by bath challenge and compared to the control group. In each treatment, five replicates of 20 fairy shrimp each were used. The potential pathogenic bacterial strains were added to separate glass aquariums (contained 2 l of water) at a final density of 1×10^4 cells ml^{-1} (LC_{50} at 48 hours was determined in a preliminary test). The fairy shrimp were observed for changes in mortality rates over two days. The pathogenic bacteria were re-isolated from the fairy shrimp. Pure strains were identified using API20E.

Experiment 2 : Screening Probiotic Bacteria for Control Pathogenic Bacteria.

Bacterial Strains and Growth Conditions

Thirteen strains of spore-forming bacteria were isolated from wild *Penaeus monodon* (*Bacillus* W120, *Bacillus* W1103 and *Paenibacillus* W803) and marine fish intestine (*Bacillus* BA, BB, BC, BF, BG, BJ, BK, BL, BKB21-1 and BSH22). The pathogenic bacterium *Aeromonas hydrophila* WS1 was isolated from infected fairy shrimp showing black disease symptoms (from experiment 1). All strains were stored as suspensions in 15% glycerol at -80°C. Before use in experiments, spore-forming bacteria and *A. hydrophila* were grown in nutrient broth (NB; Merck, Germany) at 35°C for 18–24 hours.

Inhibitory Activity of Probiotic Bacteria on *Aeromonas hydrophila* by Cross-Streak Method

Thirteen strains of spore-forming bacteria and *A. hydrophila* WS1 were cultured on nutrient agar (NA; Merck, Germany) and incubated at 35ºC for 24 hours. Inhibitory activity tests were done on NA by the cross-streak method (Lemos *et al.*, 1985). *A. hydrophila* WS1 was streaked in a line and then a spore-forming strain was streaked in a perpendicular line. Each species pair was cross-streaked in triplicate and was incubated at 35ºC for 96 hours. Inhibitory activity was observed at 24, 48, 72 and 96 hours.

Broth co-Culture of Putative Probiotic Bacteria With Pathogenic Bacteria

The spore-forming species that showed the greatest inhibition of *A. hydrophila* WS1 in cross-streak experiments were tested for antagonistic activity in a co-culture experiment. A spore-forming species and *A. hydrophila* WS1 were separately pre-cultured in 10 ml NB for 24 hours (110 rpm). NB (250 ml) was inoculated with 1×10^4 CFU/ml *A. hydrophila* WS1, together with 1×10^4 CFU/ml of spore-forming bacteria. Concentrations of spore-forming bacteria and *A. hydrophila* WS1 in co-culture were compared with monoculture controls. All flasks were incubated with shaking (150 rpm) at 35°C. All combinations were tested in triplicate. Samples were collected after 0, 24, 48, 72, 96 and 120 hours to obtain cell counts. Pseudomonas Aeromonas selective agar (glutamate starch phenol red agar; GSP agar; Merck) with penicillin G and pimaricin was used to count *A. hydrophila* WS1 cells. The number of spore-forming bacteria was calculated as the number of total bacterial cells minus the number of *A. hydrophila* WS1 cells.

Bacteria identification by 16S rDNA sequencing

Putative probiotic species that showed the greatest inhibition in cross-streak experiments were identified by 16S rDNA sequencing. Isolates were stored at -80°C as suspensions in 15% glycerol.

The universal primers (forward primer 5'-AGAGTTTGATCCTGGCTCAG -3' and reverse primer 5'-CTTGTGCGGGCCCCCGTCAATTC-3') were used for the amplification of the 16S rDNA gene fragment. Direct sequencing of the single-banded and purified PCR products (ca. 1500 bases, on 16S rDNA by the *E. coli* numbering system) (Brosius *et al*., 1981) was carried out. Sequencing of the purified PCR products was carried out with an ABI PRISM® BigDye™ Terminator Ready Reaction Cycle Sequencing Kit (version 3.0, Applied Biosystems, Foster City, California, USA). The primers 27F (5'-AGA GTT TGA TCM TGG CTC AG-3') and 518F (5'-CCA GCA GCC GCG GTA ATA CG-3') for partial sequencing, and additional 1492R (5'-TAC GGY TAC CTT GTT ACG ACT T-3') and 800R (5'-TAC CAG GGT ATC TAA TCC-3') for full length sequencing were used for sequencing of 16S rDNA. The DNA sequencing was performed on an ABI Prism® 3730xl DNA Sequence (Applied Biosystems, Foster City, California, USA).

The nucleotide sequences obtained from all primers were assembled using the Cap contig assembly program, an accessory application in the BioEdit (Biological sequence alignment editor) Program (http://www.mbio.ncsu.edu/BioEdit/BioEdit.html). A homology search was performed by using the standard nucleotide BLAST (BLASTn) from the NCBI web server (http://blast.ncbi.nlm.nih.gov/Blast.cgi) against previously reported sequences at the GenBank/EMBL/DDBJ database for determination of the nearest sequences.

Toxicity of Putative Probiotic to the Fairy Shrimp

Fairy shrimp (*B. thailandensis*) cysts were obtained from the Department of Fisheries, Faculty of Agriculture and Natural Resources, Rajamangala University of Technology Tawan-Ok, Chonburi province. The cysts were hatched and cultured in a pathogen-free environment. On the fifth day after hatching, fairy shrimp were used in experiments.

Probiotic Preparation

A pure colony of putative probiotic was inoculated into a flask containing 200 ml NB and incubated on a shaker at 200 rpm for 48 hours at 30°C. The broth was centrifuged at 2,000×*g* for 10 minutes. The bacterial pellet was resuspended in sterile water and adjusted to 1×10^7, 1×10^8 and 1×10^9 CFU/ml. These stocks were added immediately to the fairy shrimp in the glass aquarium.

Toxicity Test

The experiment consisted of four treatments, being a control group and three probiotic-treated groups (in a bath at concentrations of 1×10^4, 1×10^5 and 1×10^6 CFU/ml). Fairy shrimp were cultured at a density of 10 fairy shrimp/l. During the experiment, 2 ml of probiotic stock solution of 1×10^7, 1×10^8 and 1×10^9 CFU/ml was added to the fairy shrimp rearing water (each glass aquarium contained 2 l of water) resulting in 1×10^4, 1×10^5 and 1×10^6 CFU/ml water, respectively. Survival of the fairy shrimp was then compared to a control to determine the toxicity of the probiotic bacteria after 24, 48 and 72 hours of the experiment.

***In vivo* challenge Experiment with Fairy Shrimp and *A. hydrophila* WS1**

Fairy shrimp (*B. thailandensis*) cysts were obtained from the Department of Fisheries, Faculty of Agriculture and Natural Resources, Rajamangala University of Technology Tawan-Ok, Chonburi province. The cysts were hatched and cultured in a pathogen-free environment. On the fifth day after hatching, fairy shrimp were used in experiments.

Fairy shrimp feed

Fairy shrimp commercial formulated feed (10 g) samples were diluted in 20 ml of sterial water, filtrated by a filter bag. The aqueous feed was add 2 ml per glass aquarium (each containing 20 fairy shrimp). In the probiotic-treated group, stock of *Bacillus* W120 were prepared by the same method as the previous experiment and adjusted to a stock concentration of 1×10^7 CFU/ml . Two milliliters of bacteria suspension were mixed with aqueous feed and then fed to the fairy shrimp in each glass aquarium. Fairy shrimp in all treatments were fed twice a day.

The experiment consisted of four treatments, being a control group and three probiotic-treated groups (in a bath at concentrations of 1×10^3, 1×10^4 and 10^5 CFU/ml). Fairy shrimp were cultured at a density of 10 fairy shrimp/l. After the seventh day of feeding, fairy shrimp were challenged by immersion with *A. hydrophila* WS1 at 1×10^4 CFU/ml (LC_{50} at 24 hours). Twenty fairy shrimp were tested per replicate; each treatment consisted of five replicates. The number of dead fairy shrimp was observed and reported as the cumulative percentage mortality at 24, 48 and 72 hours post-challenge.

Study of Putative Probiotic Bacteria Survival in *B. thailandensis* Intestine

Three hundred samples of pathogen-free fairy shrimp were used in this experiment. Fairy shrimp were fed for 7 days with feed containing selected

spore-forming bacteria (1×10^3 CFU/ml per day). Addition of bacteria to feed was then discontinued, and the average concentration of selected spore-forming bacteria in the fairy shrimp intestine was determined 0, 1, 2, 3, 4, 5, 6 and 7 days after the bacterial feeding period (30 samples/day). SEM was used to confirm the results.

Scanning Electron Microscopy

Fairy shrimp intestines were excised and fixed with 2.5% w/v glutaraldehyde in 0.1 M phosphate buffer pH 7.2 for 1 hour at room temperature. After two washes with phosphate buffer, intestines were post-fixed with 1% w/v osmium tetroxide for 1 hour in the same buffer at room temperature. Post-fixed intestines were washed with phosphate buffer and dehydrated in a graded ethanol series, starting with 30% v/v and followed by 50% v/v, 70% v/v, 80% v/v and finally absolute alcohol. Intestines were dried in a critical point drier and coated with gold. The specimens were then examined with a JSM-5600 LV scanning electron microscope (JEOL, Japan).

Statistical Analysis

The data were subjected to analysis of variance followed by Duncan's multiple range test. Differences were considered significant at $P<0.05$.

Results

Experiment 1 : Pathogenic Bacterial Isolation and Identification

A total of 25 fairy shrimp individuals exhibited symptoms of black disease. Black nodules on the thoracic appendages and exoskeletons were observed (Figure 1). Based on histological study, melanin pigment which is an end product of the crustacean inflammatory response was found in the thoracic appendages. In a scanning electron microscope micrograph of the infected areas, more than three morphological types of bacteria were observed and found to adhere to the thoracic appendage, the cercopod and many areas on the exoskeleton (Figure 2). Five species of bacteria were isolated from the

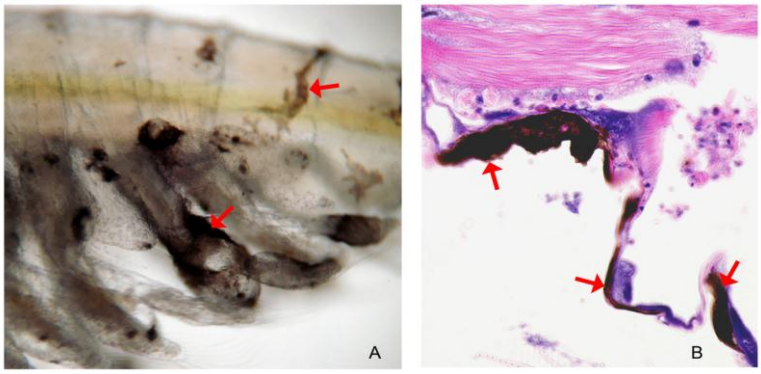

Figure 1. Clinical signs of fairy shrimp infected with bacteria. A. Black nodules on the thoracic appendage and exoskeleton (arrow). B. Histological observation showed melanization at the thoracic appendage (arrow).

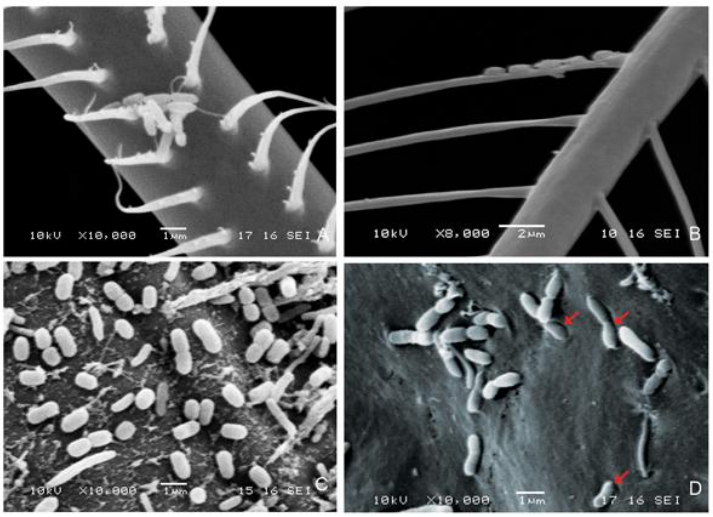

Figure 2. Scanning electron micrographs of a diseased area on a thoracic appendage of *S. thailandensis.* A. Bacteria on the thoracic appendage. B. Bacteria on a cercopod. C. Bacteria on the exoskeleton. D. Bacteria adhering to the exoskeleton (arrow).

thoracic appendages, namely, *Aeromonas hydrophila*, *Acinetobacter* sp., *Citrobacter youngae*, *Enterobacter ludwigii* and *Chryseobacterium* sp. These results indicated a high incidence of *A. hydrophila* infection, followed by *E. ludwigii*, *C. youngae*, *Acinetobacter* sp. and *Chryseobacterium* sp. (100, 40, 40, 36 and 16% prevalence, respectively).

Verification of Koch's Postulates

Immediate mortality was observed in fairy shrimp after exposure to *A. hydrophila* WS1 and *E. ludwigii* WS5 in the 24-hour study period and the clinical signs of black disease were evident. Black nodules on the thoracic appendage and exoskeleton were observed. The fairy shrimp appeared weak and lethargic after exposure to *Acinetobacter* sp. WS2, *Chryseobacterium* sp. WS3 and *C. youngae* WS4 for 24 hours and experienced increased mortality after two days. The highest cumulative mortality rate was associated with fairy shrimp challenged with *A. hydrophila* WS1 (62.00 ± 5.70%), followed by *E. ludwigii* WS5, *C. youngae* WS4, *Acinetobacter* sp. WS2 and *Chryseobacterium* sp. WS3 (51.00 ± 4.18, 35.00 ± 3.54, 10.00 ± 3.54 and 9.00 ± 4.18%, respectively).

Inhibitory Activity of Probiotic Bacteria on *Aeromonas hydrophila* by the Cross-Streak Method

Among the 13 strains of spore-forming bacteria tested against *A. hydrophila* WS1, *Bacillus* W120 showed the highest inhibition, followed by *Paenibacillus* W803. Nine strains were weakly inhibitory, namely *Bacillus* W1103, BSH22, *Bacillus* BA, BF, BG, BJ, BK, BL and BKB21-1. *Bacillus* BB and BC did not show any inhibition of *A. hydrophila* WS1.

Species Identification by 16S rDNA Sequencing

To characterize *Bacillus* W120 at the molecular level, a segment of 16S rDNA from *Bacillus* W120 was sequenced. A multiple sequence alignment was performed with DNA sequences of other *Bacillus* species obtained from GenBank. The multiple alignments showed that in the highly conserved region of 16S rDNA, a stretch at the beginning of the W120 sequence matched *B. vallismortis* rather than other *Bacillus* strains, with 99.6% similarity (Table 1). Phylogenetic trees for 1,287 bases based on 16S rDNA gene sequences were constructed using the neighbor-joining method. The tree showed clustering of WS5 sequences with *B. vallismortis* (Figure 3).

Table 1. Percent similarity of 16S rDNA compare with closely related species

	Seq ->	1	2	3	4	5
1	*B. amyloliquefaciens*	100.0	99.3	99.0	99.1	99.2
2	*B. vallismortis*	99.3	100.0	99.5	99.6	99.6
3	*B. mojavensis*	99.0	99.5	100.0	99.7	99.3
4	*B. subtilis*	99.1	99.6	99.7	100.0	99.5
5	W 120	99.2	99.6	99.3	99.5	100.0

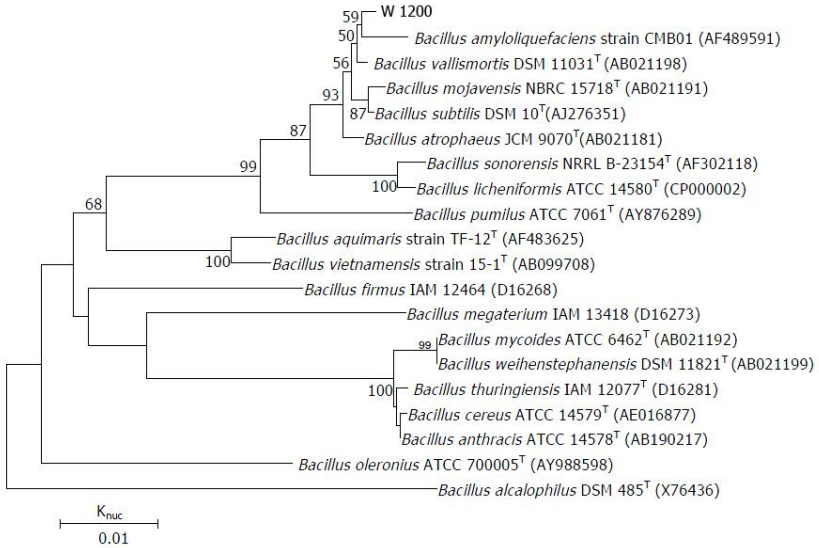

Figure 3. Phylogenetic relationships between the isolate W120 and closely related species in genus *Bacillus*. The phylogenetic trees for 1287 bases based on 16S rRNA gene sequences were constructed by Neighbor-Joining (NJ) method. *Bacillus alcalophilus* DSM 485T (X76436) was used as an outgroup. Numerals at nodes indicate bootstrap values (%) derived from 1,000 replications.

Broth co-Culture of Putative Probiotic Bacteria with Pathogenic Bacteria

A. hydrophila WS1 and *B. vallismortis* W120 grown as a monoculture increased from 1×10^4 CFU/ml to 1×10^8 CFU/ml in 24 hours. The presence of *B. vallismortis* W120 in co-culture with *A. hydrophila* WS1 reduced the growth of *A. hydrophila* WS1 from $2.53 \pm 0.43 \times 10^8$ CFU/ml (monoculture control) to $4.02 \pm 0.36 \times 10^7$ CFU/ml after 48 hours. After 120 hours of culture, *B. vallismortis* W120 inhibited growth of *A. hydrophila* WS1 from $3.53 \pm 0.76 \times 10^3$ CFU/ml (monoculture control) to $5.0 \pm 2.0 \times 10^1$ CFU/ml. In contrast, *B. vallismortis* W120 concentrations in co-culture increased to $2.38 \pm 0.13 \times 10^8$ CFU/ml after 120 hours, which was not significantly ($P>0.05$) different from the monoculture control ($2.85 \pm 0.28 \times 10^8$ CFU/ml) as shown in Tables 2 and 3.

Toxicity of Putative Probiotic to the Fairy Shrimp

For the toxicity test, fairy shrimp were cultured with probiotic bacteria suspension at concentrations of 1×10^4, 1×10^5 and 1×10^6 CFU/ml . No mortality of fairy shrimp in any treatment was observed within 72 hours of bath treatment.

Ability of Putative Probiotic Bacteria to Inhibit *A. hydrophila* WS1 Infections in Fairy Shrimp Culture

After fairy shrimp were fed with three concentration of feed containing *B. vallismortis* W120 for 7 days, they were challenged with *A. hydrophila* WS1 at 5.56×10^4 CFU/ml (LC_{50} at 24 hours). Twenty-four hours after immersion with *A. hydrophila* WS1, the mortality rate of fairy shrimp in the control group was $56.00 \pm 10.25\%$. This value was significantly ($P<0.05$) higher than that of fairy shrimp treated with *B. vallismortis* W120 at concentrations of 1×10^3, 1×10^4 and 1×10^5 CFU/ml, which had mortality rates of 36.00 ± 7.42 %, $35.00 \pm 3.54\%$ and $29.00 \pm 4.18\%$, respectively. After 48 hours, the fairy shrimp in the control group had a cumulative mortality rate of $63.00 \pm 8.37\%$. This value was significantly ($P<0.05$) higher than that of fairy shrimp treated with *B. vallismortis* W120 at concentrations of 1×10^3, 1×10^4 and 1×10^5 CFU/ml , which had cumulative mortality rates of 44.00 ± 6.52 %, $40.00 \pm 3.54\%$ and $34.00 \pm 4.18\%$, respectively. After 72 hours, the fairy shrimp in the control group had a cumulative mortality rate of $68.00 \pm 7.58\%$. This value was significantly ($P<0.05$) higher than that of fairy shrimp treated with *B. vallismortis* W120 at concentrations 1×10^3, 1×10^4 and 1×10^5 CFU/ml , which had cumulative mortality rates of 45.00 ± 7.07 %, $41.00 \pm 4.18\%$ and $34.00 \pm 4.18\%$, respectively. However, the cumulative mortality rates among the *B. vallismortis* W120-treated groups were not significantly different in any experimental period (Table 4).

Study of Putative Probiotic Bacteria Survival in *B. thailandensis* Intestine

Fairy shrimp were fed with feed containing *B. vallismortis* W120 for 7 days; bacterial feeding was then discontinued. The number of spore-forming bacteria in fairy shrimp intestine one day after discontinuation of *B. vallismortis* W120 feeding was $7.41 \pm 3.02 \times 10^3$ CFU/ individual, which was not significantly ($P>0.05$) different from the control. However, the number of spore-forming bacteria in fairy shrimp intestines decreased by about 76.64% (1,876.67 ± 965.15 CFU/individual) from the control by day 4 and by 90.01% (802.67 ± 351.17 CFU/individual) by day 7 (Table 5).

SEM revealed that *B. vallismortis* W120 could survive in fairy shrimp intestine 7 days following discontinuation of treatment with *B. vallismortis* W120. Although the number of *B. vallismortis* W120 decreased rapidly, some *B. vallismortis* W120 cells adhered to the microvilli of the fairy shrimp intestine (Figure 4).

Table 2. Concentration of *Aeromonas hydrophila* WS1 in monoculture (m) and co-culture (c) (CFU/ml)

Bacterium	(x 10^4 CFU/ml)					
	0 hour	24 hours	48 hours	72 hours	96 hours	120 hours
A. hydrophila (m)	3.60 ± 0.60^a	34500 ± 2780^a	25300 ± 425^b	123 ± 15.3^b	34.8 ± 4.31^b	0.353 ± 0.0757^b
A. hydrophila (c)	4.53 ± 0.42^a	23000 ± 1320^a	4020 ± 36.2^a	16.7 ± 3.21^a	1.83 ± 0.76^a	0.0005 ± 0.0002^a
	% decrease	33.33	84.14	86.49	94.74	98.58

Mean values within the same column sharing the same superscript character are not significantly different at P=0.05.

Table 3. Concentration of *Bacillus vallismortis* W120 in monoculture (m) and co-culture (c) (CFU/ml)

Bacterium	(x 10^4 CFU/ml)					
	0 hour	24 hours	48 hours	72 hours	96 hours	120 hours
B. vallismortis (m)	6.80 ± 1.06^a	42200 ± 4250^a	47800 ± 4310^a	40200 ± 2750^a	37300 ± 2020^a	28500 ± 2780^a
B. vallismortis (c)	6.20 ± 1.25^a	37800 ± 3690^a	41000 ± 1320^a	35500 ± 2290^a	32200 ± 1610^a	23800 ± 1260^a
	% decrease	10.28	14.29	11.62	13.84	16.37

Mean values within the same column sharing the same superscript character are not significantly different at P=0.05.

Discussion

Black disease has been reported in many species of fairy shrimp (Dierckens *et al.*, 1998). In the current study, five species of bacteria, namely, *Acinetobacter* sp. WS2, *A. hydrophila* WS1, *Chryseobacterium* sp. WS3, *C. youngae* WS4 and *E. ludwigii* WS5 were found on the thoracic appendages of diseased fairy shrimp. However, high numbers of *A. hydrophila* were found in infected fairy shrimp samples (100%) and also showed the highest cumulative mortality rate of fairy shrimp when challenged at a concentration of 1×10^4 cells ml^{-1}. *A. hydrophila* has been reported to infect fish in fresh- and warm-water fish farms worldwide and is considered a significant economic problem (Austin and Austin, 2007). It causes disease and mortality mainly in freshwater fish, both wild and cultured (Musa *et al.*, 2008).

In the current study, spore-forming bacteria, especially *Bacillus* spp., were selected for use as probiotics because this genus has an indefinite shelf life in spore form (Hong *et al.*, 2005). *Bacillus* spp. have advantages over other bacteria, as they secrete many enzymes that degrade slime and biofilms and allow *Bacillus* spp. and their antibiotics to penetrate slime layers around Gram-negative bacteria.

Table 4. Cumulative mortality percentage of fairy shrimp in the control group and fairy shrimp treated with *B. vallismortis* W120 after challenge with *A. hydrophila* WS1 for 24, 48 and 72 hours

Time after challenge with *A. hydrophila* WS1	Cumulative mortality percentage			
	Control	*B. vallismortis* W120 treatment group concentration		
		10^3 CFU/ml	10^4 CFU/ml	10^5 CFU/ml
24 hours	56.00 ± 10.25^{b}	36.00 ± 7.42^{a}	35.00 ± 3.54^{a}	29.00 ± 4.18^{a}
48 hours	63.00 ± 8.37^{b}	44.00 ± 6.52^{a}	40.00 ± 3.54^{a}	34.00 ± 4.18^{a}
72 hours	68.00 ± 7.58^{b}	45.00 ± 7.07^{a}	41.00 ± 4.18^{a}	34.00 ± 4.18^{a}

Mean values within the same row sharing the same superscript character are not significantly different at P = 0.05.

Table 5. The number of spore-forming bacteria in fairy shrimp intestine after 7 days of feeding with *B. vallismortis* W120 and after 1, 2, 3, 4, 5, 6 or 7 additional days (n=30) following discontinuation of bacterial feeding

Time after discontinuation of *B. vallismortis* W120 feeding (day)	Number of spore-forming bacteria in fairy shrimp (CFU/individual)	Percent decrease from control
0 (control)	$8{,}035.33 \pm 3{,}153.93^{a}$	0
1	$7{,}413.33 \pm 3{,}017.73^{a}$	7.74
2	$4{,}766.67 \pm 2{,}098.82^{b}$	40.68
3	$3{,}055.33 \pm 1{,}552.08^{c}$	61.98
4	$1{,}876.67 \pm 965.15^{d}$	76.64
5	$1{,}116.67 \pm 454.16^{de}$	86.10
6	953.33 ± 553.48^{de}	88.14
7	802.67 ± 351.17^{e}	90.01

Mean values within the same column sharing the same superscript character are not significantly different at P = 0.05.

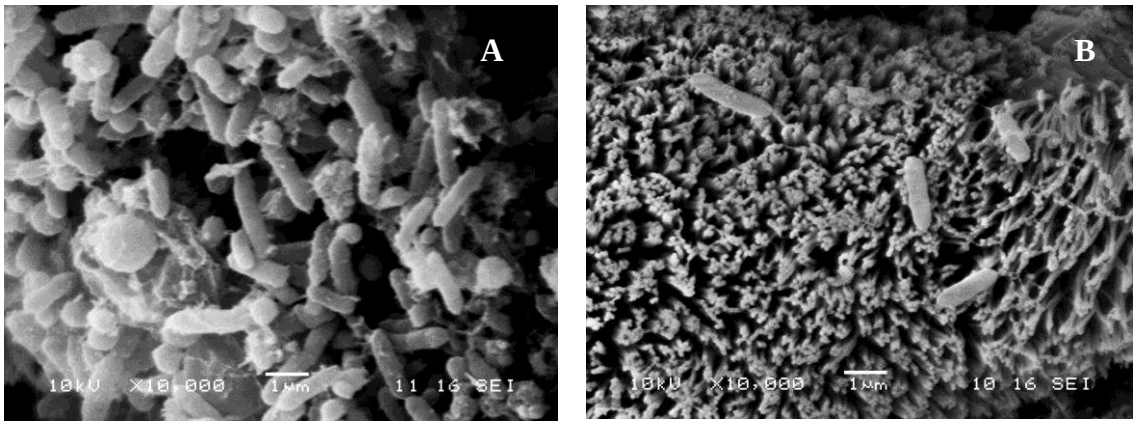

Figure 4. Examination by Scanning Electron microscope (SEM) of adherence of *B. vallismortis* W120 to fairy shrimp intestinal epithelial cells (magnification 10,000x). A. fairy shrimp intestine fed with feed added *B. vallismortis* W120. B. fairy shrimp intestine fed with feed added *B. vallismortis* W120 and stop feding add *B. vallismortis* W120 for 7 days. *B. vallismortis* W120 are seen adhering to microvilli.

Moreover, *Bacillus* spp. are unlikely to use genes for antibiotic resistance or virulence from Gram-negative bacteria (Moriarty, 1998). Previously, many species of *Bacillus*, such as *B. subtilis*, *B. licheniformis* and *B. toyoi*, were reported to act as probiotics in aquaculture (Gomez-Gil *et al.*, 2000).

In the current investigation, 13 strains selected as probiotics were examined for inhibition against *A. hydrophila* WS1, a bacterium isolated from fairy shrimp with signs of black disease. These 13 strains were screened for *in vitro* antagonism. *In vitro* antagonism is a selection criterion for potential probiotics and a typical procedure for finding probiotic or biocontrol strains in many environments (Hjelm *et al.*, 2004). Microbial populations may release chemical substances that have a bactericidal or bacteriostatic effect on other microbial populations (Verschuere *et al.*, 2000a). Many strains of *Bacillus* spp. have antagonistic activity that can reduce pathogenic bacteria populations by producing antibacterial substances, such as bacillocin 490 from *B. licheniformis*, cerein 8A from *B. cereus* 8A and thuricin 17 from *B. thuringiensis* NEB17 (Martirani *et al.*, 2002; Bizani *et al.*, 2005; Gray *et al.*, 2006).

The putative probiotic that showed the highest inhibition in the cross-streak experiments was identified as *B. vallismortis* by 16S rDNA sequencing. *B. vallismortis* is the first reported aquaculture use of this *Bacillus* species, although this species can be used for plant biocontrol of bacterial, fungal and viral diseases (Ahn *et al.*, 2002; Park *et al.*, 2007; Thanh *et al.*, 2009). *B. vallismortis* is a member of the *Bacillus subtilis* species complex and can be distinguished from *B. subtilis* only by differences in whole-cell fatty acid compositions, DNA sequences and levels of genomic DNA re-association (Roberts *et al.*, 1996; Wang, 2007; Rooney, 2009).

Competition for nutrient use was tested with the selected probiotic strain, *B. vallismortis* W120, and the pathogenic bacterium, *A. hydrophila* WS1. After 48 hours, *B. vallismortis* W120 could inhibit *A. hydrophila* WS1 and displayed *in vitro* nutrient competition against *A. hydrophila* WS1 in broth co-culture experiments. It could be hypothesized that *B. vallismortis* W120 may produce some antibacterial factors, such as antibiotics, bacteriocins, siderophores, lysozymes or proteases (Sugita *et al.*, 1998) to reduce pathogenic bacteria. Hjelm *et al.* (2004) also observed a similar phenomenon with *Roseobacter* 27-4 isolated from a range of marine and larval-rearing samples; this strain inhibited growth of *Vibrio anguillarum* 90-11-287 and *Vibrio splendidus* DMC-1 in broth co-culture experiments.

One of the important requirements of a probiotic is that it must not be pathogenic or toxic to its host. This can be determined by small-scale challenge tests of the host species using short-term baths in the bacterial suspension (Verschuere *et al*., 2000a). In the current study, the bacterium *B. vallismortis* W120 did not cause any mortality when used in short-term baths at a concentration of 1×10^6 CFU/ml. In general, *in vitro* antagonism is used to select potential probiotic bacteria, but the most important tests are to demonstrate a clear effect of the putative probiotic *in vivo* and to eliminate any strains that can cause host disease (Hjelm *et al.*, 2004). *In vivo* immersion challenge experiments with pathogenic bacteria found that fairy shrimp treated with *B. vallismortis* W120 had a survival rate significantly ($P<0.05$) higher than fairy shrimp in the control group. This result may suggest that probiotics optimize the immune system of fairy shrimp by increasing their resistance to disease, as observed in the pathogenicity challenge test. Much research has shown the ability of probiotics to reduce the mortality of aquatic animals during infection by pathogenic bacteria in laboratory experiments. For instance, *Bacillus* strain S11 could decrease the mortality of *Penaeus monodon* after challenge with pathogenic *Vibrio harveyi* D331 (Rengpipat *et al.*, 1998), and *Bacillus* strain IP5832 could decrease the mortality of turbot larvae when challenged with an opportunistic *Vibrionaceae* species (Gatesoupe, 1991). *Lactobacillus* or *Carnobacterium* isolated from rotifers (*Brachionus plicatilis*) could decrease the mortality of turbot larvae challenged with a pathogenic *Vibrio* species (Gatesoupe, 1991). *Aeromonas media* A 199 showed antagonist activity by decreasing mortality and suppressing pathogenicity in Pacific oyster larvae when challenged with pathogenic *V. tubiashii* (Gibson *et al.*, 1998). Also, on a farm scale, *Bacillus* spp. can increase the survival of aquatic animals such as prawn because they can decrease pathogenic bacterial populations in water (Moriarty, 1998).

Adhesion and colonization by probiotic bacteria in the gastrointestinal tract of the host is believed to be one of the essential features required for delivering their health benefits (Bernet *et al.*, 1994). An important aspect of probiotic bacterial function is the protection of the host gastrointestinal microenvironment from invading pathogens (Reid *et al.*, 1990). Because bacterial adhesion to tissue surfaces is important during the initial stages of pathogenic infection (Krovacek *et al.*, 1987), competition with pathogens for adhesion sites might be the first probiotic effect (Montes and Pugh, 1993). However, only a few *B. vallismortis* W120 cells remained in the fairy shrimp intestine after probiotic bacterial feeding was discontinued. Therefore, *B. vallismortis* W120 is recommended for daily use to prevent *A. hydrophila* infection during fairy shrimp culture. The presence of bacteria-producing inhibitory substances on the surface of the host intestine may constitute a barrier against proliferation of pathogens (Verschuere *et al.*, 2000a). Moreover, *Bacillus* cells also compete for space on the gut wall and, when present in high numbers, displace other bacteria (Moriarty, 1998).

In summary, *B. vallismortis* W120 has properties recommending its use as a probiotic for fairy shrimp

culture. This strain was the strongest inhibitor of *A. hydrophila* WS1 in cross-streak experiments and could decrease the number of *A. hydrophila* WS1 in NB by 84.14% after 48 hours. The survival rate of fairy shrimp fed with *B. vallismortis* W120 was higher than the control when challenged with *A. hydrophila* WS1. However, *B. vallismortis* W120 should be fed to fairy shrimp every day, because the cell population decreases by more than 50% only 3 days after bacterial feeding is discontinued.

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