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Enhanced growth of black tiger shrimp *Penaeus monodon* by dietary supplementation with *Bacillus* (BP11) as a probiotic

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Abstract

Bacillus isolate P11 (BP11), isolated from the gastrointestinal tract of the black tiger shrimp, *Penaeus monodon*, was evaluated for its potential use as a probiotic feed supplement for *P. monodon* culture. BP11, a Gram-positive spore forming bacteria, was identified as a member of the genus *Bacillus* and most likely to be an isolate of *Bacillus subtilis*, based on biochemical tests, physical morphology, and 16S rRNA gene fragment sequence analysis. BP11 is likely to be safe as a probiotic for *P. monodon* since no detectable level of antimicrobial substance or *Bacillus* diarrheal enterotoxin production was found. When the regular feed of *P. monodon* was supplemented with BP11 at ~10^9 CFU g^{-1} feed a higher shrimp growth, feed conversion ratio, survival and general health was obtained for both postlarvae (PL) shrimp in concrete tanks and in an earthen pond. In addition, and importantly, the oral administration of BP11 in the shrimp feed led to adherence to the shrimps' intestine surface with BP11 bacteria and an increased immunity to *Vibrio harveyi* 639 infection, including a reduced mortality. BP11 in dried feed had a reasonable shelf life, with viable cell counts of ~10^8 and 10^9 CFU g^{-1} remaining after 6-months storage at room temperature and 4°C, respectively.

Keywords: *Bacillus subtilis*; BP11; *Penaeus monodon*; Black tiger shrimp; Hemocyte count; Antibacterial activity; *Vibrio harveyi*

Introduction

Thailand became the world's largest farmed-shrimp exporter in 1994, exporting a total of around 30% of the world market share [1], and the value of the exported frozen and chilled shrimp from Thailand has remained high (~$ 2,500 million per year) during the last ten years (2001-2010) [2]. However, the yield per crop in most black tiger shrimp, *Penaeus monodon*, farms in Thailand began to seriously decline since 2005 with some complete losses, as seen elsewhere in SE Asia, due to the low quality of brood stocks and outbreaks of viral and bacterial infections. The resulting stunted growth and high mortality rates induced by these infections have led to a change in the major farmed shrimp species in Thailand and elsewhere away from *P. monodon* to the white shrimp, *Litopenaeus vannamei*, which now accounts for some 90% of shrimp aquaculture in Thailand and SE Asia [3,4]. Currently, the addition of substantial amounts of antibiotics or chemotherapeutics remains the current method of choice for disease control throughout the shrimp aquaculture industry. However, in addition to the imposed increased costs of such antibiotic administration, and some concern of the health and environmental affects, there is the increasing concern about the predictable spread and increase in antibiotic-resistant bacteria [5-9]. Moreover, it is not clear if the release of virulent pathogen infected aquaculture media back into the sea may lead to transient, but important, mortality in wild shrimp including short term future brood stocks for aquaculture. This has led to alternative suggestions for more sustainable and environmental friendly disease prevention, including the use of non-pathogenic probiotic bacteria [10-13]. This alternative direction of biocontrol aims to make shrimp farming more sustainable. Selection for non-pathogenic probiotic bacteria with a high specificity to the cultured shrimp host and that can provide a healthy balance of indigenous organisms in the hosts' intestines, or other benefits to the host, is the initial starting point of such an approach. However, the degree of regional (population specific) host-probiont-pathogen coevolution is unknown, and so screening of probiotic candidates should include the same brood stock populations as used for the shrimp aquaculture. In addition, the bioactive component(s) or the entire probiotic organism(s), of benefit to the health or safety of commercially farmed shrimp needs to be able to be prepared at commercial scale easily and be relatively stable to at least short-term storage (shelf life) to be of practical applicable use [12,14-20].

Although several probiotics have recently been developed for aquaculture crops, they are still not widely used. There are, however, substantial advantages for the use of probiotics in shrimp and fish aquaculture [10,11,21-26]. Among the potential bacterial probiotics for shrimp, some *Bacillus* spp. have been extensively evaluated as an additive in the feed [22-25,27-29] and in the culture water [30-32]. Several *Bacillus* spp. naturally constitute a part of the bacterial flora of the intestinal tracts of several marine taxa, including for example *B. subtilis* in fish [33-35], and *B. subtilis* and *B. pumilus* in shrimp [22,25,36]. Concurrently, *Bacillus* spp. can be isolated from shrimp culture ponds [23-25] and marine environments [37]. *Bacillus* spp. have rarely been reported as shrimp pathogens, whereas several studies have demonstrated that culturing shrimp with feed supplemented with *Bacillus* sp. reduced the subsequent mortality induced by vibriosis, in particular, some strains of *V. harveyi* [12,22,25,27]. To further study the potential of *Bacillus* spp. as a probiotic biocontrol agent in shrimp farming, and to sustain commercial black tiger shrimp aquaculture, we isolated *Bacillus* spp. from the intestines of healthy black tiger shrimp brood stocks derived from the Andaman sea, which is a different area from that previously reported [22]. Thus, this offers the potential of

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either new *Bacillus* isolates or those probiotic bacteria that are better adapted to the local shrimp-pathogen isolates of this region. Therefore, the objectives of this research were to isolate potential probiotic *Bacillus* candidates from the intestines of *P. monodon* from the Andaman sea and to evaluate their effects on the growth, survival and disease resistance of post larva shrimp *P. monodon* from the same region but reared in concrete tanks and in earthen pond, respectively.

Materials and Methods

Bacterial strains

P. monodon brood stocks (~150-200 g of ~9-12 inches each) from the Andaman sea along the West coast of Thailand were collected and their intestines were aseptically dissected out. The removed intestine was longitudinally cut and rinsed gently with sterile 0.85% (w/v) NaCl (NSS) three times and homogenized. Serial dilutions (10^{-1} to 10^{-8}) in NSS of the intestine homogenate were then plated in triplicate on tryptic soy agar (TSA) plus 1% (w/v) NaCl plates and cultured at 37°C for 24hr, and the number of colonies were counted. Isolated colonies were selected and checked for antibacterial properties against *Vibrio harveyi* 639 and *Escherichia coli* ATCC 25922 using an agar diffusion technique, as described in detail by Naclerio et al. [38].

V. harveyi 639 isolated from *Penaeus monodon* dying of luminescent disease was kindly provided by the Shrimp Culture Research Center, Charoen Pokphan Feedmill, Samutsakorn, Thailand. It was cultured in tryptic soy broth (TSB) or TSA containing 2% NaCl (w/v) at 30°C and identity was confirmed as described previously [39], Presumptive concentrations or colony-forming units (CFU ml^{-1}) of *Vibrio* spp. were determined using spread plates of thiosulfate citrate bile sucrose agar (TCBS). *Escherichia coli* ATCC 25922 was kindly provided from Department of Microbiology, Faculty of Science, Chulalongkorn University. *E.coli* was cultured on TSA and TSB at 30°C. All media were obtained from Difco, Sparks, MD, USA.

BP11 identification

The culture purity and identity of the selected isolate (BP11), which produced the greatest antibacterial performance, was routinely checked during preparation using both conventional methods and the api-20E® and api-50 CHB® Medium test kits (BioMérieux, Marcy-l'Etoile, France). The results were analyzed with the APILAB Plus software. Gram staining, spore staining, oxidase and catalase tests were performed following conventional methods and the bacterial morphology was examined as cells / spores under the microscope and as colonies on agar plates. Physiological characteristics were determined in tryptic soy broth (TSB) with various concentrations of NaCl (1-4% (w/v)) and at different pH (6-8) and temperatures (35-50°C).

Determination of the 16S rRNA nucleotide sequence

Genomic DNA of the selected isolate (BP11) was extracted following the procedure of the Geneclean II kit (Qiagen, Germany), as previously described [40]. The PCR amplification was carried out in a DNA Thermal Cycler (Perkin Elmer, USA) using the universal bacterial 16S rRNA primers: 10F 5' -AGTTTGATCCTGGCTC- 3' and 1540R 5' -AAGGAGGTGATCCAGCC- 3'. PCR reactions were carried in a total volume of 50 µl, comprised of ~1 µg of DNA template, 1µM of each primer, 1.5 mM Mg^{2+}, 200 µM dNTPs, 1.5 U *Taq* DNA polymerase in 1 x *Taq* DNA polymerase buffer. All PCR reagents were purchased from Promega, USA. The PCR conditions consisted of 35 cycles of 95°C for 1 min, 46°C for 1 min and 72°C for 1 min, followed by a final 72°C for 7 min [41]. After that, the PCR products were sequenced commercially at the BioService Unit (NSTDA, Bangkok, Thailand) using the same F and R primers for sequencing the leading and lagging strand, as used for the PCR. The consensus sequence was compared with those 16S rDNA sequences available in the NCBI GenBank database using the BLASTn search algorithm. The highest sequence identities were aligned using Clustal, checked by eye and used to compute the pair-wise sequence identity and also construct a phylogenic tree using the Neighbor Joining distance method in the PHYLIP Version 3.5. on line software, (http://evolution.genetics.washington.edu/phylip.html) (June 12, 2010).

Determination of antimicrobial substances

The evaluation of the production of antimicrobial substances by the BP11 isolate was preliminarily determined using an antimicrobial residue screening test kit (AM-Test), developed by researchers at the Center for Antimicrobial Resistance Monitoring in Food-borne Pathogens (in collaborating with WHO), Faculty of Veterinary Science, Chulalongkorn University, Bangkok, Thailand. The principle is based on the tube diffusion method and the threshold for detection limit for 18 known standard antibiotics which are in the range of > 0.001-0.1 ppm, depending upon the antibiotic. The BP11 culture broth after a 24 h (late log phase growth) and 48 h (spore forming stage) incubation at 30°C was harvested by centrifugation (at 6,230x g, 10 min at 4°C) to pellet the bacteria, and the supernatant passed through a 0.22 µm sterile filter and transferred into a tube containing semi-solid agar with spores of the Gram positive *Geobacillus stearothermophilus* and pH indicator. Milk containing antibiotic provided in test kit was used as positive control. Tubes were incubated at 65 ± 1°C for 2-2.5 h. During this incubation period any relatively small antimicrobial substances in the sample would diffuse into the agar and inhibit the growth of *G. stearothermophilus*, allowing the purple color to remain, whereas in the absence of any inhibitor a yellow color would be detected.

Determination of the enterotoxin produced by BP11 culture

Determination of the enterotoxin from filtrates of the BP11-cultured broth, were performed following the procedures according to manufacturer's instructions (TECRA® test kit; for the detection of *Bacillus* diarrheal enterotoxin (BDE); TECRA International Pty Ltd, Frenchs Forest NSW 2086, Australia). The principle of this enterotoxin test is based on the "sandwich" configuration of an enzyme-linked immunosorbent assay (ELISA).

Shrimp feed preparation

Black tiger shrimp (*P. monodon*) were fed a commercially formulated feed purchased from Grobest and Phokaphan Aquatech Corporation Co, Ltd., Thailand. The nutrition in feed by weight were as follows: protein (>35 %), lipid (>5%), fiber (>4%), moisture (<10%), and a trace of ash. For the BP11-supplemented feed, 1 kg wet weight (~100 g dry weight) of BP11 (~10^{-10} CFU g^{-1}) was thoroughly mixed with 4 kg of feed (i.e., approximately 2.5% (w/w) BP11). For this purpose, a single BP11 colony from a TSA plate was cultured in TSB at 30°C with shaking at 200 rpm for 24 h and then harvested by centrifugation at 6,230x g, 4°C for 10 min and washed three times with NSS before being weighed and re-suspended in fresh NSS. Fresh BP11 cells were then thoroughly mixed with the feed at a 1:4 (BP11: feed), and the mixture was spread out and dried in an oven for 1–2 h at 37°C. Feed was then stored in clean plastic bags at 4°C until use. Shrimp feed was prepared twice weekly, and each batch was analyzed for the level of viable BP11 cells as CFU g^{-1} by the total plate count method. This BP11-supplemented feed typically contained viable BP11 at ~3.9 x 10^8- ~ 3.6 x 10^9 CFU g^{-1}. Aliquots from each feed preparation were stored at room temperature (~ 28-32°C) and

at 4°C and assayed for viable BP11 levels (as CFU g^{-1} feed) up to six months in order to monitor their survival upon storage.

Experiment I: PL-30 shrimp culture in concrete tanks

Shrimp age of ~ post larva -15 (PL-15) were purchased from a reputable hatchery at Chachoengsao province in Thailand, and acclimatized to the PL-30 stage (0.13±0.01 g) in concrete tanks with flat bottoms (each measuring 80 x 74 x 87 cm) with a closed recirculating water system of 400 L [42]. The culture water salinity level was initially set at 20%. These PL-15 shrimp were bred from broodstock caught from the Andaman sea. After acclimatization to the PL-30 stage, healthy active shrimp were randomly assigned into the two groups: a control and probiotic treatment. Shrimp in the control group were fed with shrimp feed (CF), whilst those in the probiotic treatment group were fed with the BP11-supplemented regular shrimp feed (PF). Each treatment was comprised of three replicates, each containing 70 or 60 shrimp/tank for the two trials, respectively. Shrimp were fed three times daily at 10% body weight and after 90 days of culture the survival (%) and total live weights (g) were determined.

Experiment II: PL-50 shrimp culture in net cages in earthen pond

Hatchery reared *P. monodon* from PL-50 (0.23±0.06 g), obtained from the same shrimp hatchery and broodstock as the PL-15 above, were first acclimatized in the cement tank. Shrimp were then preselected for healthy active shrimp and then randomly assigned to the CF- and PF- treatments, as detailed above, and stocked in ten 1.5 m^2 net cages (1.0 mm mesh) at 100 shrimp/cage. The net cages were all placed at a 1.3 m water depth in a single 1000 m^2 surface area earthen pond (typically 1.5 m deep) along an access walkway. Cages were suspended 20 cm above the pond bottom and extended 40 cm above the water surface. Five cages contained the control (CF) shrimp, while the PF shrimp were housed in the remaining five cages. Individual cages were separated by a distance of approximately 1 m. A row of five cages were separated by a distance 1.5 m on opposite sides of the walkway for two rows. Two rows of walkways were separated by a distance of one meter. Pond water salinity was ~6-7‰ during the dry season of Thailand and was aerated with a closed-system operation. All shrimp were fed three times daily at 10% body weight in the net cages for 80 days.

Intestinal microbial investigation of CF- and PF shrimp intestines

The CF- and PF shrimp of 80 days culture in an earthen pond were randomly selected and kept on ice. Their gastrointestinal tracts were dissected out. The intestines were longitudinally cut and rinsed vigorously with sterile NSS three times before fixation and a portion was fixed in 2.5% (w/v) glutaraldehyde in 0.1 M phosphate buffer pH 7.2 for 2 hr. After fixation these samples were then processed following conventional procedures, as reported previously [43], and examined by scanning electron microscopy (SEM) using a Jeol JSM-5410LV (Japan) electron microscope.

Water samples collection: Water samples were collected from the center of each tank (20 ml) and net cage (200 ml) from each treatment for determination of the viable BP11 cells every 15 or 20 days. Water quality measurements included the dissolved oxygen, ammonium, nitrite and phosphate levels plus the temperature, pH and salinity, and were evaluated as described by Strickland and Parsons [44].

Vibrio *harveyi* challenge tests: After 90 and 80 days, shrimp cultured in concrete tanks and in an earthen pond, respectively were randomly taken and challenged with *Vibrio harveyi* 639. The PF- and CF shrimp were subjected to static, disease challenge tests using the luminescent bacteria *V. harveyi* 639 at ~10^7 CFU ml^{-1}, according to Austin et al. [10] and modified from Rengpipat et al. [22]. Each challenge included 12 tanks with a 2 x 2 factorial design. Treatments were the PF- and CF shrimp challenged with *V. harveyi*, and unchallenged PF- and CF shrimp. Treatments were performed in triplicate containing 30 and 25 shrimp per tank for shrimp cultured from concrete tanks of Trial I and II, respectively. Triplicate replications of each treatment, using 20 shrimp per tank, for shrimp cultured from the net cages in an earthen pond.

During *V. harveyi* challenge tests, no water was exchanged and the water quality parameters and shrimp survival were measured every 2 d. The cumulative number of dead shrimp was recorded each day. Shrimp that died following *V. harveyi* 639 exposure were dissected and the hepatopancreas-intestine and heart muscle were removed, cut and fixed in Davidson's fixative solution for 24 h and then processed for paraffin sectioning by standard techniques. Tissue sections (8 µm thick) were prepared and processed for haematoxylin and eosin staining (H & E) followed by indirect immunoperoxidase staining using first the anti-*Vibrio* VH3-3H murine monoclonal antibody [45] and secondly the horseradish peroxidase conjugated goat-anti-mouse (GAM-HRP) secondary antibody, diluted to 1:1000 in 10% (v/v) normal calf serum in PBS, as reported previously [45]. A positive immunoreactivity was visualized as a deep brown coloration against the pink and purple background of the H & E staining. In addition, the bacteria isolated from the hepatopancreas-intestine and heart muscle of each shrimp, were purified and identified using Gram staining, oxidase and motility tests as described previously [39], and were compared with the original *V. harveyi* 639 culture to confirm the similarity to the *V. harveyi* strain.

Haemolymph collection: For determination of the total haemocyte count and antibacterial activity before and after challenge with *V. harveyi*, the haemolymph from shrimp that were cultured in the net cages in the earthen pond was collected from the ventral-sinus cavity of each shrimp using a 26-gauge needle and a 1-ml syringe containing modified KC-199 medium (K-199 medium plus Hepes 2.38 g l^{-1}, supplemented with 5% (w/v) L-cysteine as an anticoagulant [46]).

Total haemocyte count: A 100 µl aliquot of the haemolymph, collected from three randomly selected shrimp per tank, was diluted into 0.4 ml of modified KC-199 medium and mixed gently, and then the haemocytes were counted using a haemocytometer under a light microscope at 400x magnification, and calculated as cells ml^{-1} of haemolymph.

Antibacterial activity: One hundred microlitre of the shrimp haemolymph was added to 1.4 ml of ice cold, sterile Van Harrevald's salt solution (VHS) [47], mixed and then centrifuged at 600x g for 10 min at 4°C. The plasma supernatant was collected and sterilized by filtration (millipore membrane filter, 0.45 µm pore size) for subsequent assays for antibacterial activity. *V. harveyi* 639 was cultured in TSB supplemented with 2% (w/v) NaCl overnight at 37°C. The concentration was adjusted to ~10^4 CFU ml^{-1}, collected and washed in 2% (w/v) NSS by centrifugation at 92,006x g for 15 min at 4°C. The *V. harveyi* 639 suspension and shrimp plasma (100 µl each) were mixed in a sterile test tube and incubated for 1 hr at 37°C. Three 50 µl aliquots were taken from each tube and spread on thiosulfate-citrate-bile-sucrose (TCBS) agar plates to estimate the viable bacterial numbers as CFU ml^{-1} after incubating at 37°C for 24 hr. For the positive control, *V. harveyi* 639 were incubated with VHS. The percentage inhibition was calculated as

reported elsewhere [48], where; %inhibition = 100 - (mean CFU ml^{-1} sample/mean CFU ml^{-1} positive control) x 100

Statistical analysis: The effect of BP11 on the shrimp growth and survival were evaluated using student's t-test [49] with $p<0.05$ as the confidence level for significant differences. Completely randomized design was carried out to determine for immune indices and disease resistance after shrimp challenged by *V. harveyi* 639.

Results

BP11 identification and its characteristics

Of the 245 bacterial isolates obtained from screening the intestines of freshly caught *P. monodon* broodstocks from the Andaman sea, BP11 isolate produced the greatest antimicrobial activity against *V. harveyi* 639 and *E. coli* (data not shown), and so was selected for further evaluation.

BP11, a Gram-positive rod bacteria with a cell size of ~0.55-0.75 x 2.5-3.5 µm (width x length), can form a central spore (Figure 1), and gave good growth in TSB containing 1 - 4% (w/v) NaCl; at temperature of 35-50°C and pH of 6-8. The best growth was found in TSB with 2% (w/v) NaCl at 45°C and a pH of 6.5 (data not shown). The BP11 isolate was identified using the api-20E® and api-50 CHB® Medium test kits, where, after analysis with the APILAB Plus software, the percentage similarity of BP11 to *Bacillus* subtilis was found to be 99.9%. Moreover, sequence analysis of the 1491 bp partial 16S rRNA gene fragment from BP11 (GenBank accession code HM585370) exhibited the highest sequence identity to those sequences annotated as *B. subtilis* or *B. amyloliquefaciens* (>99% sequence identity), supported by a NJ distance based phylogenetic tree which grouped BP11 within the *B. subtilis*/*B. amyloliquefaciens* clade (data not shown). Thus, the biochemical and morphological analysis combined with the 16S r-RNA based molecular analysis place BP11 as a potential isolate of *B. subtilis* within the Firmicutes bacterial division.

Antimicrobial and enterotoxin detection

In contrast to the positive control (milk containing antibiotics) that showed a purple color, a yellow color was detected in the AM-test for filtrates of BP11 broth. Thus, no antimicrobial residue was detected in the BP11 broth after 24 and 48 hr of culture. In addition no BDE was detected in the BP11 broth culture by the TECRA test kit.

PL-30 and PL-50 shrimp culture in concrete tanks and in earthen pond

Water quality: The water quality values, during the two experiments each, for the shrimp cultures in the concrete tanks and in the earthen pond were similar for dissolved oxygen (~10 and 6.1-7.7 mg l^{-1}), ammonium (0.0-0.5 and 0.0 mg l^{-1}), nitrite (0.0-0.5 and 0.1mg l^{-1}) and phosphate (0.1-0.2 and <0.1-0.25 mg l^{-1}), but slightly different for the pH (6.5-7.0 and 7.7-8.4), temperature (26-27 and 28.4-31.4°C) and salinity (20-23 and 6.1-7.2%). However, all of these water quality parameters were considered safe for shrimp culture [42,50,51]. BP11 counts of ~10^2-10^3 CFU ml^{-1} were found in all culture water taken from the concrete tanks or net cages of shrimp fed with BP11-supplemented feed (data not shown).

Experiment I: PL-30 Shrimp growth in concrete tanks

The average live weights of the CF shrimp in the concrete tanks after 90 days of culture for the two separate trials (6.17 ± 0.61 g and 6.99 ± 0.19 g) were significant different ($p < 0.05$) from those of the PF shrimp (7.48 ± 0.23 g and 8.94 ± 0.43 g) (Table 1).

Experiment II: PL-50 shrimp growth in the net cages in the earthen pond

The influence of BP11 on PL-50 shrimp growth and survival over 80 days was evaluated by simulating to a certain extent the conditions used in commercial shrimp culture using 1.5 m^2 surface area net cages, located in a 1,000 m^2 surface area earthen pond. The average live weights of the PF shrimp (13.74±0.77 g) in the net cages after 80 days culture were significantly different from that of the CF shrimp (12.86±0.38 g) (Table 2). In addition, the survival of PF-group shrimp (76.77 ± 3.78 %) was higher than that in the CF shrimp (65.2 ± 7.60) (Table 2). The food conversion ratio and productive index in the PF-group shrimp were better than those in the CF shrimp (Table 2).

Intestinal microbial investigation of CF- and PF shrimp intestines

After culture for 80 days in the earthen pond, both the CF- and PF shrimp were randomly selected. The intestines were dissected out for sample preparation and examination by SEM to detect adherent bacteria on the intestinal wall. No bacteria conforming to the morphology of the BP11 was detected on the surface of the intestine of the CF shrimp

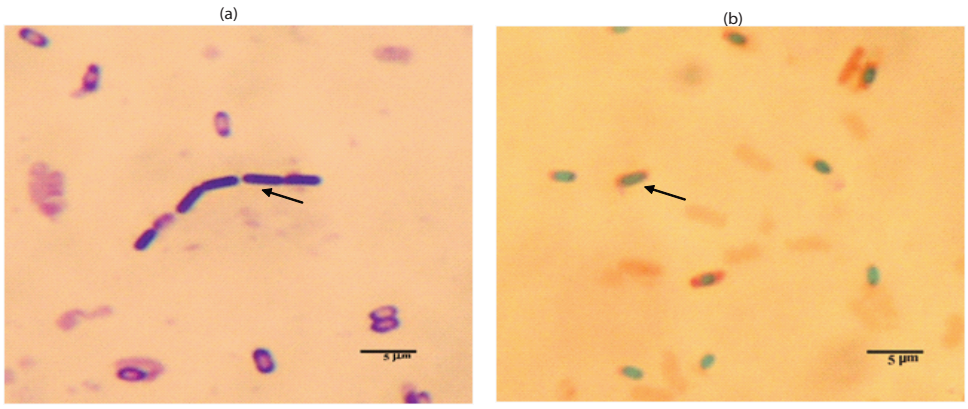

Figure 1: Light microscope images (x 1,000 magnification) of the Gram-positive rods and spores of BP11. (a) The cells, sized ~0.55-0.75 x 2.5-3.5 µm (width x length) and (b) the central spore of BP11 as pointed by arrow.

Experiment I	Control (CF)	Probiotic (PF)
Trial I (n = 210)		
Average shrimp live weight (g) on day 90 of culture (n = 30)	6.17 ± 0.61	7.48 ± 0.23*
Average daily gain (mg)	68.13 ± 6.73	82.68 ± 2.52*
Survival (%)	67.14 ± 6.54	84.29 ± 3.78*
After challenge by *V. harveyi*:		
Cumulative mortality (%) on day 4	55.0 ± 5.29	28.33 ± 3.06*
Trial II (n = 180)		
Average individual live weight (g) on day 90 of culture (n = 30)	6.99 ± 0.19	8.94 ± 0.43*
Average daily gain (mg)	77.21 ± 2.07	98.89 ± 4.74*
Survival (%)	61.11 ± 2.48	63.89 ± 1.47
After challenge by *V. harveyi*:		
Cumulative mortality (%) on day 5	56.0 ±5.33	17.78± 2.04*
Cumulative mortality (%) on day 9	100	44.0 ±3.53*

*Indicates a significant difference ($p < 0.05$) between the control (CF) and probiotic (PF) treatment groups.
Average daily gain; Total weight per days of culture

Table 1: Survival and average live weight of shrimp after 90 days culture in concrete tanks fed with (PF) or without (CF) BP11 supplemented feed, and their cumulative mortality after challenge by *Vibrio harveyi* 639.

Parameters	Control(CF)	Probiotic(PF)
Total live weight (kg)	4.56	5.33
Average individual live weight (g) on day 80 of the culture (n = 100)	12.86 ±0.38	13.74 ± 0.77*
FCR[(1)]	2.02	1.78
Survival (%)	65.2±7.6	76.8±3.78
PI[(2)]	184	287.5

*Indicates a significant difference ($p < 0.05$) between the control (CF) and probiotic (PF) shrimp
[(1)]Food conversion ratio; dried weight of ingested feed per live weight of produced shrimp
[(2)]Productive index; Shrimp weight x %survival x 100 per FCR x age

Table 2: Survival and total live weight of shrimp after 80 days of culture in net cages in an earthen pond fed with (PF) or without (CF) BP11 probiotic supplement in the feed (Experiment II).

(Figure 2a and b), whereas many rod-shaped bacteria were observed on certain surface area of some intestine portions from PF shrimp (Figure 2c and d). Concurrently, BP11 counts from the total bacteria counts (~10^6-10^7 CFU g^{-1} intestine of either CF-or PF shrimp, data not shown) were < 10^1-10^2 and ~10^5 -10^6 CFU g^{-1} intestine for the CF- and PF shrimp, respectively (data not shown), which supports the possible presence of BP11 on the surface of the PF shrimp's intestines.

Longevity of BP11 in dried feed

Viable BP11 counts in the BP11 supplemented feed were found to be ~ 10^9 and ~10^8 CFU g^{-1} of feed after storage for 6 months at 4°C and room temperature (28-32°C), respectively. No changes in the physical morphology or the biochemical properties of BP11 were detected upon storage at either temperature for 6 months. Moreover, a clear probiotic affect of BP11, including the growth and vibriosis resistance enhancement in *P. monodon* (*in vivo*), was evident after storage at -18°C for 2 years, where for example the data of Table 2 is derived from the use of a 2 year old storage culture of BP11,

V. harveyi challenge test

The shrimp cultured in the concrete tanks for 90 days were collected and tested for disease resistance to a *V. harveyi* challenge by external exposure. It was found that the cumulative shrimp mortality was significantly lower in the PF shrimp than in the CF shrimp after external challenge with *V. harveyi* 639 for 4 or 5 days. After 9 days, 100% cumulative mortality was found in the CF shrimp compared to only 44% for the PF shrimp (Table 1). Thus, the BP11 supplemented feed appeared to afford (prophylactic) protection to the *P. monodon* shrimp against *V. harveyi* 639 induced mortality.

Likewise, when PL-50 shrimp collected from the net cage culture after 80 days in the earthen pond were challenged by *V. harveyi* 639

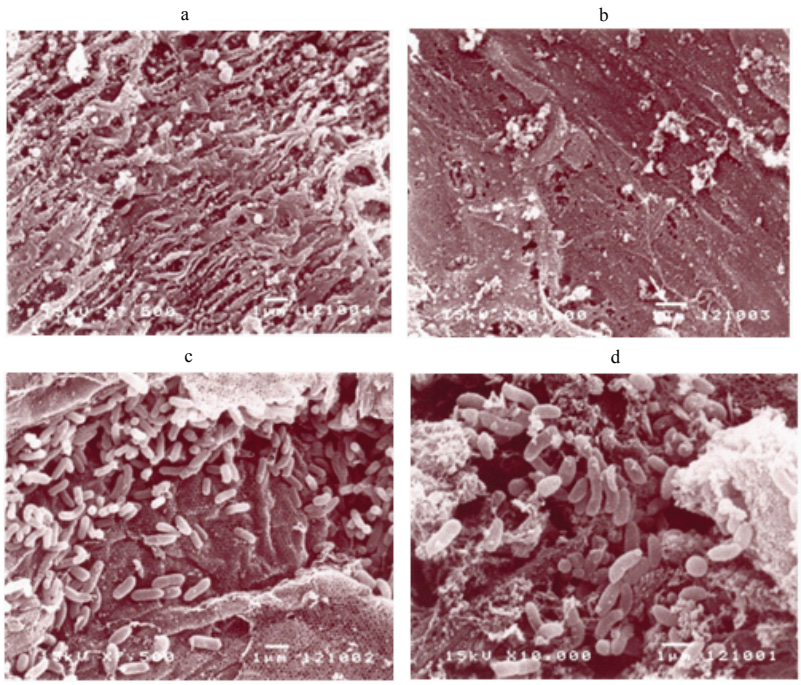

Figure 2: Representative SEM images of intestinal bacteria of 80-day earthen pond cultured shrimps' intestines (a, b) without (CF) or (c, d) with BP11 supplementation and shown at (a, c) x 7,500 or (b, d) x 10,000 magnification.

Parameters	Before *V. harveyi* challenge		After *V. harveyi* challenge	
	Control (CF)	Probiotic (PF)	Control (CF)	Probiotic (PF)
Total haemocytes (x10^7 cells ml^{-1})	1.32 ± 0.69	1.62 ± 0.19	0.35 ± 0.24	0.11 ± 0.10
Antibacterial activities (% inhibition)	17.3 ± 4.0[a]	39.0 ± 3.9[b]	63.7 ± 8.1[c]	71.1 ± 5.6[c]
Cumulative mortality (%); day 5	_	_	81.7 ± 16.1	67.7 ± 10.4

Data are shown as the mean + 1 SD, and are derived from nine shrimp per treatment. Means within a row followed by a different superscript letter are significantly different ($p < 0.05$).

Table 3: Mean immunity indices values and cumulative mortality (%) of shrimp fed with (PF) or without (CF) BP11 supplement in the feed in net-cage culture in an earthen pond, before and after challenge with *Vibrio harveyi* 639 for 5 days.

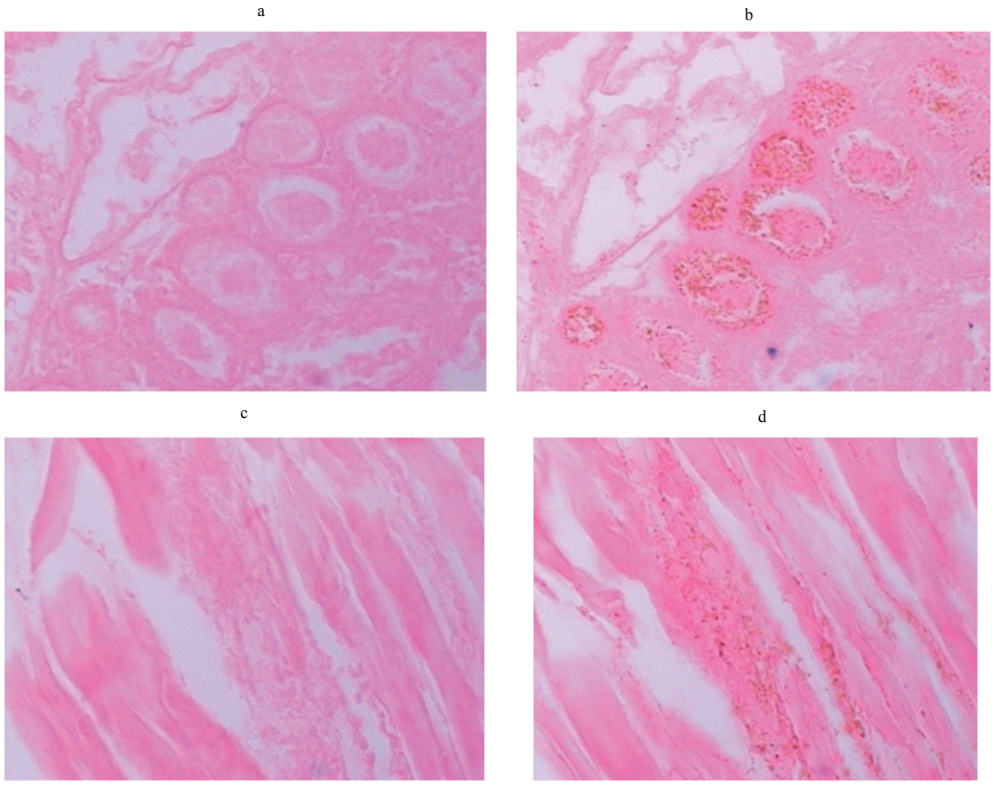

Figure 3: Representative immunohistochemical detection of *V. harveyi* infected tissues from dead *P. monodon* following exposure to *V. harveyi* 639. The (a,b) hepatopancreas and (c,d) heart muscle after staining by haematoxylin and eosin(H & E) while the right panels (b,d) in each case were also stained with indirect immunoperoxidase using the VH3-3H monoclonal antibody as a probe. Thus, brown dots show the presence of *V. harveyi* 693. Images shown are representative from three independent shrimp and examined under light microscope (x 1,000 magnification).

for 5 days, significantly lower cumulative mortality was observed in the PF shrimp than in the CF shrimp (Table 3). Survival was 100% in unchallenged control shrimp.

Those PL-30 and PL-50 shrimp from the concrete tanks and earthen pond, respectively, that had died after exposure to *V. harveyi* 639 were checked for evidence of internal *V. harveyi* infection by immunohistochemistry of the hepatopancreas-intestines and muscle tissues using the *V. harveyi* 639 specific VH3-3H monoclonal antibody (Figure 3), and by standard microbiological examination (*V. harveyi* counts were ~10^5 - 10^6 CFU g intestine, data not shown). In all cases *V. harveyi* infection was detected in the examined internal tissues. No shrimp mortality was detected among unchallenged CF-and PF shrimp.

Immunity indices: Only the PL-50 shrimp from the net cages in the earthen pond were collected for monitoring their immunity indices during the *V. harveyi* challenge test over 5 days. Before *V. harveyi* challenge the total haemocyte level and the antibacterial activity, from the PF shrimp were numerically greater than those of the CF shrimp (Table 3), although only the antibacterial activity was significantly different between the two groups. After *V. harveyi* challenge both the CF- and PF shrimp significantly increased their antibacterial activities and decreased the number of circulating haemocytes, but a significantly greater increase in the antibacterial activity upon *V. harveyi* challenge was noted for the PF shrimp. Also, the total circulating haemocyte numbers of the PF shrimp were lower than those of the CF shrimp.

Discussion

BP11 is one of the natural bacterial flora found in the intestines of black tiger shrimp from the Andaman sea off the Thai coast and was found not to produce any detectable amount of antimicrobial substances or BDE in TSB based liquid culture. BP11 was identified as being a member of the genus *Bacillus* and most likely as an isolate of *B. subtilis*, which is not known as a human or animal pathogen, nor is it toxigenic like some other members of this genus [52]. *B. subtilis* has also been authorized in the list of additives for feeding stuffs published by

the European Union Commission [53]. BP11 was preliminarily selected as a probiotic candidate after its inhibitory effect on *Vibrio harveyi* 639 growth had been detected *in vitro* (data not shown). BP11 level in freshly dried feed ~10^8 and 10^9CFU g^{-1} was still found after storage for 6 months at room temperature and 4°C, respectively, showing that BP11 is fairly stable in accord with the fact that *Bacillus* species can resist extreme environments and can produce spores, as can be seen for the BP11 isolate (Figure 1b). This affords them a greater survival, including a longer shelf life in feed, than other groups of bacteria during storage [54]. In addition, after storage at -18°C for 2 years, BP11 was found to still be able to cause growth enhancement in *P. monodon* (*in vivo*), as shown in Table 2, supporting the efficacy of BP 11 to induce beneficial effects to hosts, to be able to retain their probiotic properties after feed processing, and to display a sufficient bacterial survival level during storage. These are all important properties for consideration as a good probiotic, as suggested previously [55,56].

More than 15 years after the first finding of *Bacillus* S11 (BS11) as a potential probiotic for black tiger shrimp [22], we here isolated *Bacillus* P11. Although both BS11 and PP11 have been identified as isolates of *Bacillus* subtilis, their cell size, physical appearance of their colonies on agar plates and the optimal growth conditions of BS11 and BP11 are different, suggesting they are different strains. It is thus unclear if this represents stable probiotic-shrimp relationships between different host populations or a change over time due to environmental changes.

After 80 days of being fed BP11 supplemented feed (PF shrimp) in an earthen pond, BP11 bacterial counts in the shrimp intestines were found to be ~10^5- 10^6 CFU $g^{-1}$of shrimp intestines, and likely BP11 (cells conforming to *Bacillus* BP11 morphology) were found on the intestine's surface by SEM analysis (Figure 2c and d). Meanwhile, very rare detection of bacteria on the intestines of the CF shrimp (fed with regular feed) was observed by SEM analysis (Figure 2a and b), though total bacteria counts ~10^6-10^7 CFU g^{-1} intestine of either CF-or PF shrimp were determined (data not shown). This finding supports the ability of BP11 to adhere to the mucosal surface of the shrimp's intestines more than other bacterial flora (Figure 2), which supports our previous finding of BS11 on shrimp intestines' surface using Green fluorescent protein (GFP) as a monitoring system [57]. The benefits of probiotics to the host have been reported as providing an interfering substance to inhibit pathogens, produce useful enzymes or increase the uptake of useful nutrients, provide a competitive exclusion effect on pathogens and help beneficial immune induction [23,25,27,58-60]. In our case, using the principle of balancing an indigenous microorganism *in situ* along the intestinal tract, BP11 may possibly function as a protective barrier against pathogens, perhaps by competitive exclusion preventing pathogens like *V. harveyi* from gaining occupation and becoming a major member of the intestinal flora as mentioned earlier [12,14-17, 19,20,25,61]. However, they may also produce enzymes to digest and absorb nutrients and thus lead to healthy shrimp. That BP11 adhered to the surface of the shrimp intestinal wall better than other bacterial flora may due to specific interaction (recognition) between the shrimp host and probiotic bacteria. All the above beneficial effects of probiotic bacteria on the shrimp are not mutually exclusive and could occur simultaneously. In addition, the lysis of the probiotic bacteria could release peptidoglycan, an immunogen that can induce the immune responses of the shrimp, as suggested previously [62-65].

The yield of probiotic fed shrimp (PF group) in the concrete tanks were significantly higher than those of the control shrimp (CF group). However, before BP11 can be recommended for use in commercial farms, confirmation was sought by evaluation of its effects in net caged shrimp in an earthen pond that more closely simulates an actual commercial culture environment. Our results still confirmed the same positive outcome of shrimp fed BP11, including the prophylactic response towards protection from vibriosis. In addition, *B. subtilis* is known to be a non- to low- virulent species and requires a high number of bacteria to cause disease in humans [66]. Therefore, in conclusion, BP11 could be a good probiotic candidate for black tiger shrimp, *P. monodon*, as an alternative to antibiotics or chemical agents, and may lead to a more sustainable and safe commercial shrimp culture. However, regarding the recognition as safe for human consumption, risk assessment of BP11 should be further confirmed.

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