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Penaeus monodon larvae can be protected from *Vibrio harveyi* infection by pre-emptive treatment of a rearing system with antagonistic or non-antagonistic bacterial probiotics

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Abstract

This study shows that the disease resistance and survival rate of *Penaeus monodon* in a larval rearing systems can be enhanced by supplementing with antagonistic or non-antagonistic probiotics. The antagonistic mode of action of *Pseudomonas* MCCB 102 and MCCB 103 against vibrios was demonstrated in larval mesocosm with cultures having sufficient concentration of antagonistic compounds in their culture supernatant. Investigations on the antagonistic properties of *Bacillus* MCCB 101, *Pseudomonas* MCCB 102 and MCCB 103 and *Arthrobacter* MCCB 104 against *Vibrio harveyi* MCCB 111 under *in vitro* conditions revealed that *Pseudomonas* MCCB 102 and MCCB 103 were inhibitory to the pathogen. These inhibitory properties were further confirmed in the larval rearing systems of *P. monodon*. All these four probionts significantly improved larval survival in long-term treatments as well as when challenged with a pathogenic strain of *V. harveyi* MCCB 111. We could demonstrate that *Pseudomonas* MCCB 102 and MCCB 103 accorded disease resistance and a higher survival rate in *P. monodon* larval rearing systems through active antagonism of vibrios, whereas *Bacillus* MCCB 101 and *Arthrobacter* MCCB 104 functioned as probiotics through immunostimulatory and digestive enzyme-supporting modes of action.

Keywords: crustacean larviculture, probiotics, *Vibrio harveyi*, *Penaeus monodon*, *Pseudomonas*, *Bacillus*, *Arthrobacter*

Introduction

Globally, penaeid shrimps rank sixth in terms of quantity and second in terms of value among all the taxonomic groups of aquatic animals cultivated (FAO 2006). The intensification of shrimp culture brought in a host of diseases, among which white spot disease caused by the white spot syndrome virus (WSSV) and luminescent vibriosis caused by *Vibrio harveyi* have been the scourge for nearly two decades (Austin & Zhang 2006; Pang, Zhang, Zhong, Chen, Li & Austin 2006; Defoirdt, Boon, Sorgeloos, Verstraete & Bossier 2007). Luminescent vibriosis is particularly severe during the larval stages and sporadically causes temporary closure of economic activities in shrimp larviculture clusters. The successful completion of the larval cycle requires appropriate technologies to maintain the healthy microbial balance of the system, thus preventing the outbreak of luminescent vibriosis. Traditional technology followed intensive water exchange and/or application of disinfectants and antimicrobials to complete the larval cycle

successfully (Holmstrom, Graslund, Wahlstrom, Poungshompoo, Bengtsson & Kautsky 2003). However, the emergence of antibiotic-resistant pathogens with the threat of gene transfer to human pathogens, and environmental issues pertaining to wastewater discharge led to the development of alternative biological tools to manage diseases. In this context, manipulation of microbial balance in the larval rearing tanks by introducing non-pathogenic probiotic strains isolated from native environment became a promising technology (Liao, Su & Chang 2001; Vine, Leukes & Kaiser 2006; Defoirdt *et al.* 2007; Guo, Liu, Cheng, Chang, Lay, Hsu, Yang & Chen 2009). Early definitions of probiotics included only the organisms that, when delivered through feed, improved the health and survival of the hosts (Fuller 1989). However, the definition was expanded to include also the organisms that created a favourable environment for the growth and well-being of the host animals by their transience through the gastrointestinal tract or merely by their presence in the water (Gatesoupe 1999; Verschuere, Rombaut, Sorgeloos & Verstraete 2000). Survival of fish and shellfish larvae is increasingly reliant on the incidental microbiota of the eggs, rearing water and the live food organisms supplemented during the first feeding stages (Singh, Lakshmanaperumalswamy & Chandramohan 1989; Olafsen 2001). Therefore, manipulating the aquatic environment with known probiotic strains can positively influence the well-being of shrimp larvae during larviculture (Verschuere, Rombaut, Huys, Dhont, Sorgeloos & Verstraete 1999; Verschuere *et al.* 2000).

The demand for probiotics has resulted in the flooding of the market with preparations having little or no scientific documentation (Decamp, Moriarty & Lavens 2008). Although different microorganisms have been identified as potential probiotics, their efficacy at the field level has not been elucidated completely (Balcázar, Blas, Ruiz-Zarzuela, Cunningham, Vendrell & Múzquiz 2006). There is a need to understand the host–microbe interactions and their influence on the resident flora so that probiotic applications can be tailored to the situation and not empirically as is the current practice. Such an approach is hampered by the sparse information on the mechanism of action of the candidate strains and the inability of the organism to produce bioactive molecules at the required concentration under *in vivo* conditions. The evidence gained so far shows that the probable mechanism of action of probiotics *in vitro* includes secretion of inhibitory substances, digestive enzymes and stimulating the non-specific immune system of the animals (Balcázar *et al.* 2006; Kesarcodi-Watson, Kaspar, Lategan & Gibson 2008; Hai, Buller & Fotedar 2009). The inhibitory molecules such as nisin of *Lactobacillus lactis*, pediocin of *Pediococcus acidilactici*, siderophore-like molecules and pyocyanin of *Pseudomonas* spp. are well characterized (Baron & Rowe 1981; Guerra & Pastrana 2002; Price-Whelan, Dietrich & Newman 2006). The biotechnological applications of antagonistic organisms producing inhibitory compounds are very promising in aquaculture (Vaseeharan & Ramasamy 2003; Hjelm, Bergh, Riaza, Nielsen, Melchiorsen, Jensen, Duncan, Ahrens, Birkbeck & Gram 2004; Ravi, Musthafa, Jegathammbal, Kathiresan & Pandian 2007), because of their ability to control the pathogenic bacterial population in a system without affecting the total chemical or biochemical balance of the environment (Kesarcodi-Watson *et al.* 2008; Tinh, Yen, Dierckens, Sorgeloos & Bossier 2008). Previously, we isolated from shrimp culture systems *Pseudomonas* and *Micrococcus* spp. (reclassified as *Arthrobacter* spp. in this study), which inhibited the growth of *Vibrio* spp. under *in vitro* conditions through inhibitory compounds (Jayaprakash, Pai, Anas, Preetha, Philip & Singh 2005; Vijayan, Singh, Jayaprakash, Alavandi, Pai, Preetha, Rajan & Santiago 2006). The inhibitory compound of *Pseudomonas* spp. was identified as pyocyanin and the conditions for production were optimized (Preetha 2006; Preetha, Jayaprakash, Philip & Singh 2007). In the present study, we investigated the replication of microbial antagonism observed *in vitro* under *in situ* conditions in *Penaeus monodon* larval mesocosms and the effects of antagonistic and non-antagonistic probiotics on larval survival when challenged by *V. harveyi* MCCB 111.

Materials and methods

Bacteria

Four bacterial isolates, *Pseudomonas* MCCB 102 (PS 102) (Vijayan *et al.* 2006), *Pseudomonas* MCCB 103 (Preetha *et al.* 2007), *Arthrobacter* MCCB 104 (Jayaprakash *et al.* 2005) and *Bacillus* MCCB 101, were tested for antagonism against 87 isolates of *V. harveyi*. All isolates belonged to the Microbial Culture Collection – Bacteria (MCCB) at the National Centre for Aquatic Animal Health (NCAAH), Cochin University of Science and Technology (CUSAT). Isolates of *V. harveyi* were from postlarva and water samples of *P. monodon* hatchery systems including larvae afflicted with luminous bacterial disease. All isolates

were cryopreserved in 20 g L^{-1} salinity ZoBell's marine broth 2216E (ZB) with 10% glycerol at −80 °C. Working cultures were maintained in 20 g L^{-1} salinity ZoBell's marine agar (ZA) slants at 28 ± 1 °C. The identity of all the isolates was ascertained by sequencing a 1500-bp fragment of the 16S rRNA gene from the genomic DNA using the primers NP1F 5′-GAGTTTGATCCTGGCTCA-3′ and NP1R 5′-ACGGCTACCTTGTTACGACTT-3′, complementary to the conserved regions at the 5′- and 3′- ends of the 16S rRNA gene corresponding to positions 9–27 and 1477–1498 of the *Escherichia coli* 16S rRNA gene (Reddy, Aggarwal, Matsumoto & Shivaji 2000). The 16S rRNA gene was amplified from bacterial DNA (50 ng) in a total volume of 25 μL containing 10 pmol of each of the two primers, and 200 μM each of dNTPS, 1 U Taq DNA Polymerase (New England Biolabs, Ipswich, MA, USA) in the buffer provided by the supplier. The amplification consisted of 35 cycles of 94 °C for 20 s, 58 °C for 20 s, 72 °C for 90 s and a final extension at 72 °C for 10 min. The amplified products were gel purified (QIAEX II, Qiagen, Valencia, CA, USA) and cloned into pGEMT Easy plasmid vector (Promega, Madison, WI, USA) following the supplier's instructions. The clones were sequenced using the primer walking service of Microsynth AG, Switzerland. The sequences obtained were submitted in GenBank under the accession numbers EF062514 (MCCB 102), EF053508 (MCCB 103), EF062509 (MCCB 101), EU402968 (MCCB 104) and EU404191 (MCCB 111). The identities of all strains at the genus level were ascertained at 98% homology to the type strain for which a maximum score was obtained in the BLAST algorithm (Altschul, Madden, Schäffer, Zhang, Zhang, Miller & Lipman 1997). The isolate MCCB 104 identified earlier as *Micrococcus* based on the phenotypic features (Jayaprakash *et al.* 2005) was reassigned to the genus *Arthrobacter* based on the 16S rRNA gene sequence obtained in this study.

In vitro antagonism assay and co-culture experiments

Antagonism of the four putative probionts was tested using disc diffusion assay (Jayaprakash *et al.* 2005). Briefly, each of the bacterial isolates were grown in 20 g L^{-1} salinity ZB on a shaker (120 rpm) at 28 ± 1 °C for 5 days. ZoBell's marine agar plates were swabbed with 0.5 mL overnight grown culture (1 OD at A_{600}) of the target bacterial isolates (87 isolates of *V. harveyi*). Aliquots (20 μL) of the four bacterial cultures were dispensed onto the 6 mm diameter discs prepared from Whatman no. 1 filter papers separately in triplicate. Antagonism among the four putative probionts was also tested. The plates were incubated for 24 h at 28 °C and the formation of a zone of clearing around the discs was considered to be a positive indication of inhibitory activity. The zone of inhibition around the discs was recorded after 24 h using the HiAntibiotic Zone Scale (Himedia, Mumbai, Maharashtra, India).

Antagonism of the cell-free supernatant of the four putative probiotic bacteria was also tested. The four cultures were grown in ZB for 5 days on a shaker (120 rpm) at 28 ± 1 °C. Cells were pelleted by centrifugation (10 000 *g*, 4 °C, 10 min), the pH of the supernatant was adjusted to 7.0 and then passed through a 0.2 μm pore-size cellulose-acetate membrane filter (Sartorius, Goettingen, Germany). Inhibitory activity against 87 *V. harveyi* isolates and the four probionts was detected using the disc diffusion method as described above.

In vitro co-culture experiments of MCCB 102 and MCCB 103 with *V. harveyi* MCCB 111 were carried out independently following the method of Gram, Melchiorsen, Spanggaard, Huber and Nielsen (1999). They were pre-cultured separately in ZB at 28 ± 1 °C on a shaker at 120 rpm overnight. From the above cultures, *V. harveyi* MCCB 111 was inoculated in 100 mL ZB to obtain an initial cell count of approximately 10^3 CFU mL^{-1}, whereas the initial levels of MCCB 102 and MCCB 103 in those flasks were 0, 10^4, 10^5, 10^6 and 10^7 CFU mL^{-1} respectively. The flasks were incubated at 28 ± 1 °C on a shaker (120 rpm), and samples (1 mL) were withdrawn at 24-h intervals for determination of the cell count. Counts of *V. harveyi* were estimated using H&L medium (Hugh and Leifson 1953). Tubes containing 4 mL of H&L medium were inoculated with 1 mL aliquots of a serially 10-fold diluted culture and overlayed with sterile liquid paraffin and incubated at 28 ± 1 °C for 24 h. The fermentative growth of *V. harveyi* MCCB 111 led to a change in the pH of the medium. The highest dilution, which showed growth and pH change, was used to calculate the count of *V. harveyi* MCCB 111 in the sample (1×10^n, where n is the highest dilution that showed a fermentative reaction) and expressed as $\log_{10}$ CFU mL^{-1}. The cell count of *Pseudomonas* MCCB 102 and MCCB 103 was monitored on pseudomonas isolation agar (PIA) (Himedia) using the spread plate method. In a separate experiment, overnight-grown *Pseudomonas* MCCB 102 and MCCB 103 were inoculated in 100 mL ZB to obtain an initial

concentration of 10^4 CFU mL^{-1}. The flasks were incubated at 28 ± 1 °C on a shaker (120 rpm), and samples (6 mL) were withdrawn at 24-h intervals for determination of the cell count and the concentration of pyocyanin secreted. The cell counts of *Pseudomonas* MCCB 102 and MCCB 103 were measured as mentioned above. Pyocyanin was assayed by extracting 5 mL culture supernatant with 3 mL of chloroform. This was then re-extracted in 1 mL 0.2 N HCl to give a pink-coloured solution. The absorbance of this solution was measured at 520 nm and the concentration in micrograms of pyocyanin produced per millilitre of culture supernatant determined by multiplying the absorbance at 520 nm by a factor 17.072 following Essar, Eberly, Hadero and Crawford (1990). Also, the ^{1}H NMR spectra of the pyocyanin secreted by *Pseudomonas* MCCB 102 and 103 were recorded on a Bruker AMX 400 (Fallender, Switzerland) high-resolution multinuclear FT-NMR spectrometer operating at 400 MHz and compared with standard pyocyanin. $CdCl_3$ was used as the solvent and tetramethylsilane (TMS) was used as the internal standard.

Co-cultures of MCCB 101 and MCCB 104 with *V. harveyi* MCCB 111 were carried out individually at the same initial cell numbers as above. Counts of the pathogen were monitored by withdrawing daily 1-mL samples that were serially diluted 10-fold and 0.2 mL aliquots spread plated on TCBS agar and ZA plates. The plates were incubated at 28 ± 1 °C for 24 h and the colonies obtained on TCBS were counted and expressed as $\log_{10}$ CFU mL^{-1} of *V. harveyi* in the co-culture. Both the Gram-positive bacteria (MCCB 101 and MCCB 104) did not grow on TCBS agar and could be easily differentiated from *V. harveyi* on ZA based on colony characteristics. MCCB 104 formed yellow non-luminescent colonies while those of MCCB 101, apart from being non-luminescent, were rough and white. All co-culture combinations were maintained in duplicate and the experiments were repeated twice.

In situ effect of long-term treatment with the putative probionts on *P. monodon* larvae and postlarvae

Groups of 2000 *P. monodon* larvae at Protozoea I stage were introduced into five 100-l fibre-reinforced plastic (FRP) tanks and reared for 38 days until metamorphosis to postlarva (PL)-30 at a commercial shrimp hatchery. The effect of the four putative probiotics MCCB 101, MCCB 104, MCCB 102 and MCCB 103 on the health and survival of *P. monodon* larvae was assessed independently. One group ($n = 2000$) was maintained as the control without any probiotic exposure. All probiotics were pre-cultured in ZB at 28 ± 1 °C for 5 days and added to the rearing water every 2 days to obtain a final cell count of 10^6 CFU mL^{-1}. The larvae, protozoea I to mysis III, were maintained on a diet of *Chaetoceros* spp. (at 80 000–13 0000 cells mL^{-1}) and thereafter from PL-1 on freshly hatched *Artemia* nauplii (at 0.5–1 individual mL^{-1}). Water in the tanks was increased to 100 L gradually until conversion to PL-1, following which 25–30% water was exchanged daily. The physico-chemical parameters of the rearing water such as, total ammonia, nitrite, pH, salinity and temperature were monitored regularly following standard methods (Clesceri, Greenberg & Eaton 1998). Three water and larval samples were drawn from each tank once in 3 days for monitoring the total heterotrophic bacterial population (TPC) and total *Vibrio* count (TVC). Water samples (1 mL) were serially diluted 10-fold and 0.2-mL was spread on ZA and TCBS agar plates in duplicate. Ten larvae from each group were washed gently in sterile seawater to remove loosely adhering particles and macerated in 1 mL of sterile seawater, serially diluted 10-fold and 0.2 mL spread on ZA and TCBS in duplicate. All plates were incubated at 28 ± 1 °C for 24–72 h and those having 30–300 colonies were taken to estimate the bacterial counts, which were expressed as $\log_{10}$ CFU mL^{-1} and CFU larvae^{-1} for water and larval samples respectively. The health of the postlarva (PL-30) was assessed (20 from each group) and scored by microscopic observation for features such as muscle opaqueness, deformities, size variation, gut content, colour and condition of the hepatopancreas, epibiont fouling, intestinal persistalsis and muscle to gut ratio as per the FAO guidelines (FAO 2003). A formalin stress test was also conducted by subjecting 100 postlarvae from each group to 100 ppm formalin for 1 h and then monitoring them in normal rearing water for another 3 h. The final survival in each group was taken when the larvae reached PL-30.

Challenge test with *V. harveyi*

Three groups of 30 postlarvae (PL-24) from each of the treatments (probiotic treated and control) above were transferred to plastic containers (3 L capacity). From the control group, two sets of 30 PL each were maintained. They were acclimatized for 24 h, following which an overnight culture of the luminescent bacterium *V. harveyi* MCCB 111 was added to water to obtain 10^6 CFU mL^{-1}. This isolate has been proven to cause mortality in penaeid shrimp larvae (Patra

& Mohamed 2003). One group (probiotic untreated) of 3 × 30 PL was maintained as negative control and not challenged. An *ad libitum* feeding regime with freshly hatched *Artemia* nauplii and probiotic addition as mentioned above was continued. The total vibrio and luminescent bacterial counts of water and larvae were assessed on ZA and TCBS agar as mentioned in the previous section on the first and the third day when the experiment was concluded.

Statistical analysis

The results of the long-term treatment experiment (when the larvae reached PL-30) were compared statistically using χ^2 test. The effects of the probiotics on the total vibrio population, luminescent bacteria and survival after challenge with *V. harveyi* were assessed using ANOVA, and the means of the different treatment groups were compared using the least significant difference (LSD) multiple range test at a 5% level of significance.

Results

In vitro antagonism assay and co-culture experiments

In the antagonism assay, the culture and cell-free supernatants of the putative probionts *Pseudomonas* MCCB 102 and MCCB 103 inhibited all the 87 isolates of *V. harveyi* tested (Fig. 1). However, the zone of inhibition obtained with the culture and cell-free supernatants of MCCB 104 was turbid (a thin layer of cell growth is observed) against all the *V. harveyi* isolates (Fig. 1). The cells isolated from the turbid zone exhibited normal growth in ZB as well as on ZA plates, which indicated that the antagonistic molecule produced by *Arthrobacter* MCCB 104 was not inhibitory to *V. harveyi* MCCB111. *Arthrobacter* MCCB 104 did not inhibit *Bacillus* MCCB 101, *Pseudomonas* MCCB 102 and MCCB 103. Neither the culture nor the cell-free supernatant of *Bacillus* MCCB 101 was inhibitory to any of the isolates tested.

The antagonism exhibited by *Pseudomonas* MCCB 102 and MCCB 103 against *V. harveyi* MCCB 111 was further confirmed in the co-culture assay. *Pseudomonas* MCCB 102 inhibited *V. harveyi* MCCB 111 in co-culture when the initial cell count of the putative probiont was $> 10^5$ CFU mL^{-1} (Fig. 2a). However, similar inhibition of *V. harveyi* MCCB 111 by *Pseudomonas* MCCB 103 was obtained only at initial cell counts $> 10^6$ CFU mL^{-1} of the putative probiont (Fig. 2b). This difference could be attributed to the variation in the concentration of pyocyanin secreted by MCCB 102 and MCCB 103 (Fig. 2c and d). Although, both MCCB 102 and 103 begin to produce pyocyanin from the late log phase and peak in the stationary phase, it was consistently higher in the culture supernatant of MCCB102. Lower cell counts of *Pseudomonas* MCCB 102 and MCCB 103 allowed initial growth of *V. harveyi* MCCB 111 ostensibly due to insufficient pyocyanin in the culture media. Its concentration, however, increased upon continued incubation thus never allowing the cell densities of the pathogen to reach close to that of the control. In contrast, *V. harveyi* MCCB 111 growth was not inhibited in co-cultures with *Bacillus* MCCB 101 and *Arthrobacter* MCCB 104 even at 10^8 CFU mL^{-1} initial densities of the probionts (Tables 1 and 2). Growth of the probionts was not affected by *V. harveyi* MCCB 111 at any stage in the co-cultures (data not shown). Proton NMR spectra of pyocyanin secreted by *Pseudomonas* MCCB102 (top) and 103 (middle) shows a good similarity to that of the pure pyocyanin sample (Fig. 3).

In situ effect of long-term treatment with the putative probionts on *P. monodon* larvae and postlarvae

The *in vitro* antagonism of vibrios observed with *Pseudomonas* MCCB 102 and MCCB 103 was replicated during the *in situ* long-term treatment experiment. The total vibrio population was significantly lower ($P < 0.05$) in the rearing water and larvae of the tanks that were supplemented with *Pseudomonas* MCCB 102 and MCCB 103 (Figs 4 and 5). Notably, vibrios were not detected in the larval samples from these tanks until metamorphosis to PL-8. In agreement with the *in vitro* results, there was no effect on the TVC in the earing water and larvae of the groups supplemented with *Bacillus* MCCB 101 and *Arthrobacter* MCCB 104 compared with that of the untreated control (Figs 4 and 5). In all treatment groups, luminescent bacteria could not be detected at any stage during the experiment. The mortality of *P. monodon* larvae was significantly lower ($\chi^2 = 228.9$, $P < 0.05$) in all probiotic treatment groups compared with that in the untreated control (Fig. 6). The highest TVC (Figs 4 and 5) and maximum cumulative mortality (Fig. 6) were observed in the tanks without any probiotic application, indicating the pathogenicity of vibrios. Interestingly, larval survival was significantly and comparatively higher ($P < 0.05$) in the group that

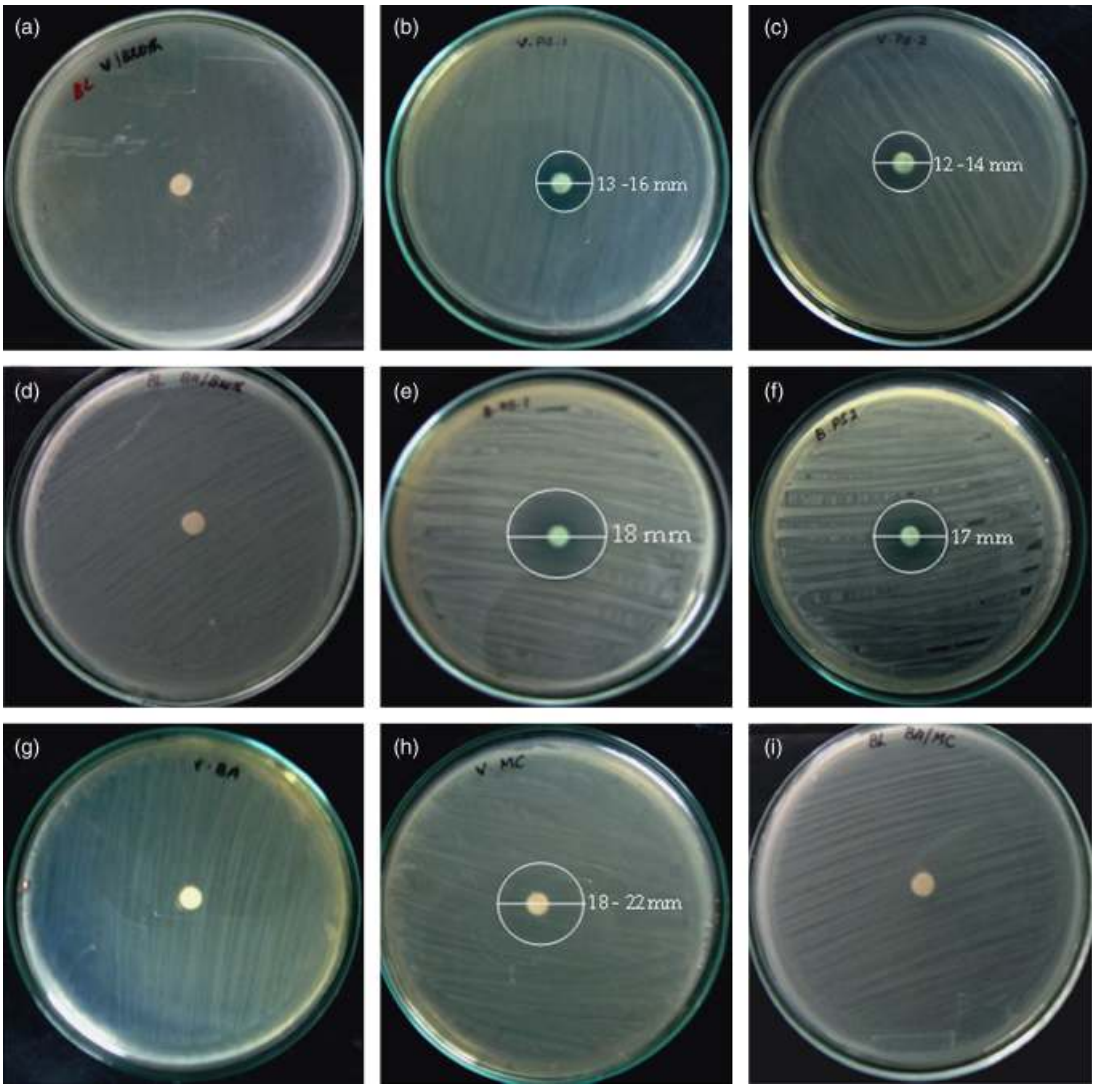

Figure 1 Antagonistic activity of cell-free supernatants of the putative probionts against *Vibrio harveyi* MCCB 111 (b, c, g, h) and between them (e, f, i) in a disc diffusion assay. (a) ZB against *V. harveyi* MCCB 111. (b) *Pseudomonas* MCCB102 against *V. harveyi* MCCB 111. (c) *Pseudomonas* MCCB103 against *V. harveyi* MCCB 111. (d) ZoBell's marine broth against *Bacillus* MCCB 101. (e) *Pseudomonas* MCCB102 against *Bacillus* MCCB 101. (f) *Pseudomonas* MCCB103 against *Bacillus* MCCB 101. (g) *Bacillus* MCCB 101 against *V. harveyi* MCCB 111. (h) *Arthrobacter* MCCB 104 against *V. harveyi* MCCB 111. (i) *Arthrobacter* MCCB 104 against *Bacillus* MCCB 101.

received *Arthrobacter* MCCB 104 compared with the other probionts tested. When 20 larvae from each group were examined under the microscope, they were all transparent, with their guts full, dark hepatopancreas, high intestinal peristalsis, no fouling or deformities on the exoskeleton or gills, muscle: gut ratio at the sixth abdominal segment $> 3{:}1$ and a uniform size (%CV $< 15\%$) (Table 3). Larvae in all treatment groups did not show any signs of weakness when subjected to formalin stress test, indicating the good health of the larvae (Supporting information Table S2). In conclusion, all the four bacterial putative probionts tested could significantly improve *P. monodon* larval survivals, and the application of the pseudomonads MCCB 102 and 103 resulted in reducing the *Vibrio* population during the early larval stages.

Challenge with *V. harveyi* MCCB 111

We demonstrated the ability of the four putative probionts to protect *P. monodon* postlarva from *Vibrio*

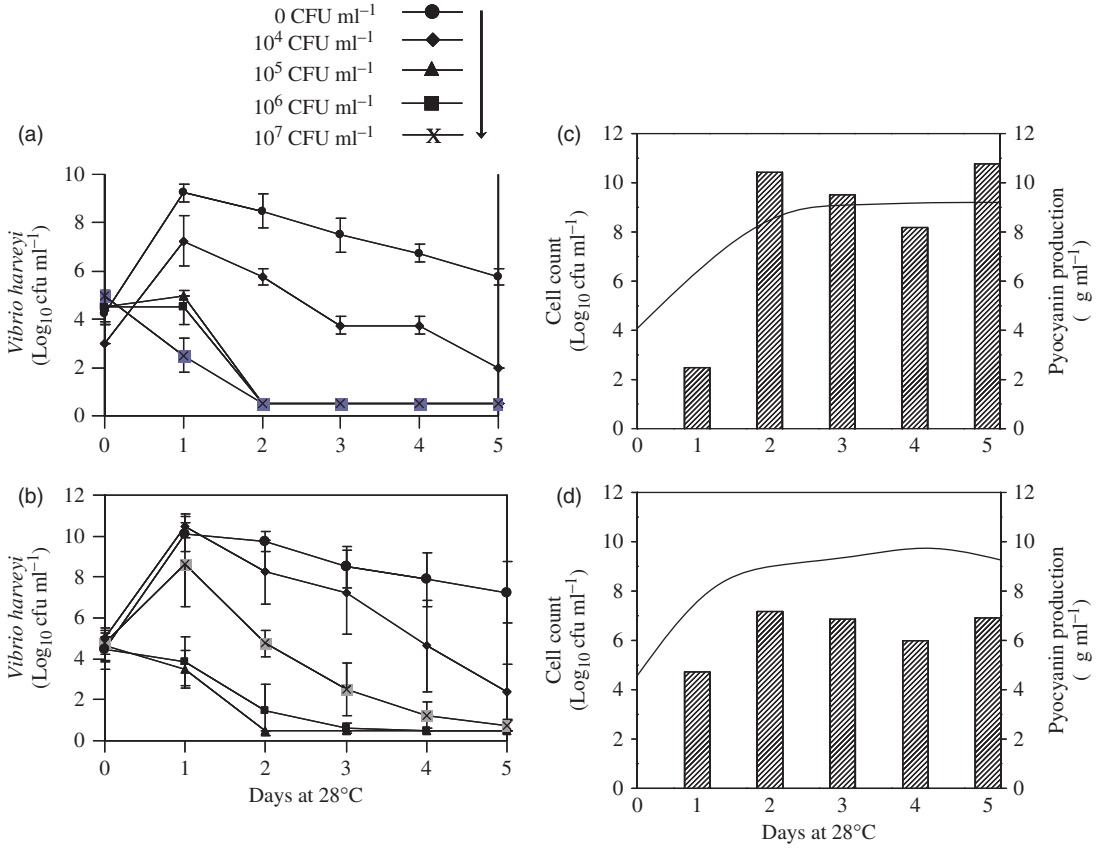

Figure 2 Growth of *Vibrio harveyi* in coculture at increasing cell densities (no. cells, 10^4, 10^5, 10^6 and 10^7 CFU mL^{-1}) of *Pseudomonas* MCCB 102 (a); *Pseudomonas* MCCB 103 and the production of pyocyanin (column) by *Pseudomonas* MCCB 102 (c); *Pseudomonas* MCCB 103 (d) at different growth stages (lines).

Table 1 Growth of *Vibrio harveyi* MCCB 111 over 5 days at different cell densities of *Arthrobacter* MCCB 104 under co-culture

Arthrobacter **MCCB104 initial cell density (CFU mL^{-})**	***Vibrio harveyi*** **LB three counts (mean log$_{10}$ CFU mL^{-1})**		
	0 h	**24 h**	**120 h**
10^8	3.38 ± 0.09	8.12 ± 0.40	8.43 ± 0.13
10^7	3.27 ± 0.25	9.01 ± 0.33	8.74 ± 0.37
10^6	3.30 ± 0.25	9.49 ± 0.21	8.74 ± 0.54
10^5	3.51 ± 0.52	9.48 ± 0.17	8.84 ± 0.37
10^4	3.64 ± 0.41	9.51 ± 0.23	9.11 ± 0.25
No MCCB 104	3.83 ± 0.23	9.57 ± 0.26	9.13 ± 0.19

± Standard error.

Table 2 Growth of *Vibrio harveyi* MCCB 111 over 5 days at different cell densities of *Bacillus* MCCB 101 under co-culture

Bacillus **MCCB101 initial cell density (CFU mL^{-})**	***Vibrio harveyi*** **LB three counts (mean log$_{10}$ CFU mL^{-1})**		
	0 h	**24 h**	**120 h**
10^8	3.91 ± 0.03	8.77 ± 0.01	8.57 ± 0.03
10^7	3.87 ± 0.04	9.71 ± 0.02	9.15 ± 0.05
10^6	3.93 ± 0.01	9.61 ± 0.06	9.30 ± 0.06
10^5	3.99 ± 0.09	9.72 ± 0.01	9.21 ± 0.12
10^4	4.04 ± 0.09	9.74 ± 0.03	9.37 ± 0.03
No MCCB 101	3.97 ± 0.02	9.80 ± 0.01	9.10 ± 0.15

infection by challenging with the opportunistic pathogen *V. harveyi* MCCB 111 (Fig. 7). On completion of larval rearing with and without the putative probiotics, postlarva (PL30) were challenged with 10^6 CFU mL^{-1} *V. harveyi* MCCB 111 and the cumulative mortality was monitored after 3 days. Significantly higher mortality was observed in the larvae reared without any probiotics, confirming the pathogenicity of *V. harveyi* MCCB 111 (Fig. 7a). The mortality was significantly lower ($P < 0.05$) in the groups that

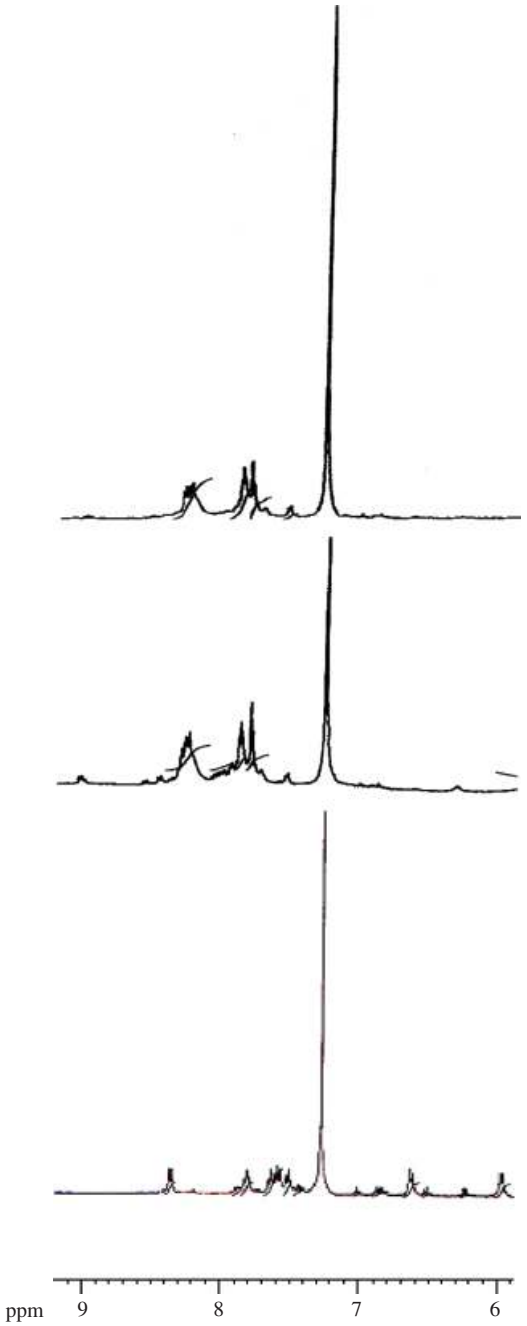

Figure 3 Comparison of proton nuclear magnetic resonance spectra for pyocyanin secreted by *Pseudomonas* MCCB 102 (top), *Pseudomonas* MCCB 103 (middle) and a pure pyocyanin (bottom).

were previously treated with the probiotics. Luminescent bacterial counts (LBC) were significantly lower ($P < 0.05$) in the groups that received pre-emptive treatment with *Pseudomonas* MCCB 102 and MCCB 103, indicating that the antagonistic compound present in the rearing water negatively impacted the *V. harveyi* population (Fig. 7b). No luminescent bacteria were detected in any of the larval samples that received the probionts while they were recovered from the control that was not under probiotic treatment (Fig. 7c). This showed that the *V. harveyi* MCCB 111 had indeed infected the larvae and was the cause of larval mortality. *Vibrio harveyi* was estimated in all samples from the luminous bacterial counts obtained from ZA enumeration plates. Interestingly, their counts obtained on TCBS were always one log lower than those on ZA and therefore only the ZA counts were considered. In conclusion, the larvae pre-emptively treated with the four putative probionts were effectively healthier and could successfully ward off *V. harveyi* infection. It was further confirmed that the application of *Pseudomonas* MCCB 102 and MCCB 103 to the rearing water could effectively reduce the *V. harveyi* population.

Discussion

We could demonstrate that the disease resistance and survival rate of *P. monodon* in larval rearing systems can be promoted by the supplementation of probiotics. The *in vitro* antagonism of the four probionts assessed using the disc diffusion and co-culture experiments showed that *Pseudomonas* MCCB 102 and MCCB 103 effectively inhibited the growth of *V. harveyi* MCCB 111. The viability of *V. harveyi* in the turbid zone of clearance obtained with *Arthrobacter* MCCB 104 indicated that it was neither bactericidal nor bacteriostatic. The inhibition of *V. harveyi* MCCB 111 by *Pseudomonas* MCCB 102 and MCCB 103 could be attributed to the production of the antagonistic compound pyocyanin and siderophores, secreted into the culture broth (Preetha 2006; Vijayan *et al.* 2006; Preetha *et al.* 2007). Pyocyanin and other phenazine compounds secreted by fluorescent pseudomonads mediated inhibition of other bacteria due to their unusual redox properties, which resulted in an enhanced production of hydroxide (OH^-) and super oxide (O_2^-) radicals, resulting in oxidative damage, DNA damage and lipid peroxidation (Baron, Terranova & Rowe 1989; Muller 2002; O'Malley, Reszka, Spitz, Denning & Britigan 2004; Angell, Bench, Williams & Watanabe 2006; Mavrodi, Blankenfeldt & Thomashow 2006). It can also interact synergistically with the siderophore pyochelin and sequester the micronutrient iron from the environment thereby giving *Pseudomonas* spp. a survival advantage (Coffman,

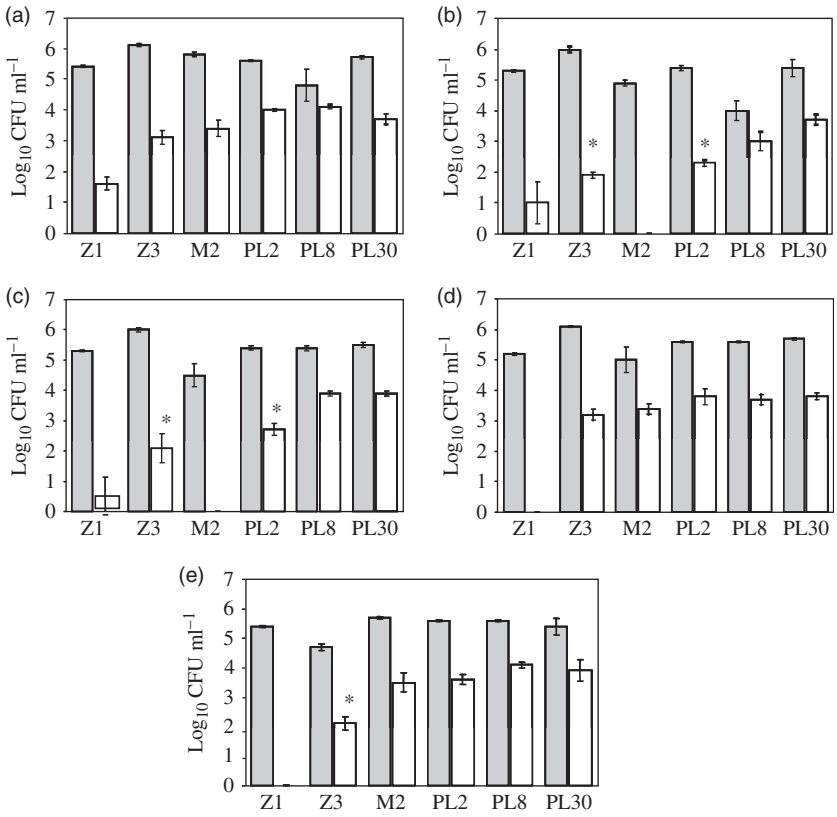

Figure 4 Progression of total heterotrophic bacterial (grey bars) and vibrio population (white bars) in the larval rearing water of *Penaeus monodon* with and without a probiotic treatment at different stages of growth. (a) *Bacillus* MCCB 101, (b) *Pseudomonas* MCCB 102, (c) *Pseudomonas* MCCB 103, (d) *Arthrobacter* MCCB 104, (e) control (no treatment); Z1, zoea 1; Z3, zoea 3; M2, mysis 2; PL2,postlarva 2; PL8, postlarva 8; PL30, postlarva 30; *values significant at $P < 0.05$.

Cox, Edeker & Britigan 1990; Britigan, Roeder, Rasmussen, Shasby, McCormick & Cox 1992). Co-cultures allow the study of the interaction between two organisms in terms of antagonism and competition for micronutrients particularly under nutrient-limiting conditions (Gram *et al.* 1999). The production of pyocyanin by the *Pseudomonas* isolates used in the co-cultures apparently gave them a competitive advantage over *V. harveyi* MCCB 111 mediated by the killing of the pathogen. However, *Bacillus* MCCB 101 and *Arthrobacter* MCCB 104 could not gain competitive advantage over *V. harveyi* MCCB 111 because the former did not produce any antagonistic molecules and the antagonistic property exhibited by the latter *in vitro* was so weak too have control over the pathogen.

A major challenge in the *in situ* demonstration of the antagonistic mode of action of probiotics in aquaculture systems is to ensure sufficient concentration of an antagonistic compound in the culture supernatant. Previously, we optimized the fermentation conditions of *Pseudomonas* MCCB 102 and MCCB 103 to maximize the secretion of pyocyanin in the culture supernatant (Preetha *et al.* 2007) and in the present study, *in situ* antagonism was successfully achieved through regular application of the culture. It is evident that *Pseudomonas* MCCB 102 and MCCB 103 could not only control the vibrio population but could also significantly improve larval survival in shrimp larval mesocosms. On the other hand, significant differences in TVC were not observed in rearing water and larvae of tanks supplemented with *Bacillus* MCCB 101 and *Arthrobacter* MCCB 104. The production of phenazine compounds by fluorescent pseudomonads is profoundly influenced by environmental factors, a plausible reason for the lack of sustained activity when applied in the environment (van Rij, Wesselink, Chin-A-Woeng, Bloemberg & Lugtenberg 2004), which can be overcome by regular replenishment of the probiont. Therefore, the application of probiotics with antagonistic mode of action is a viable

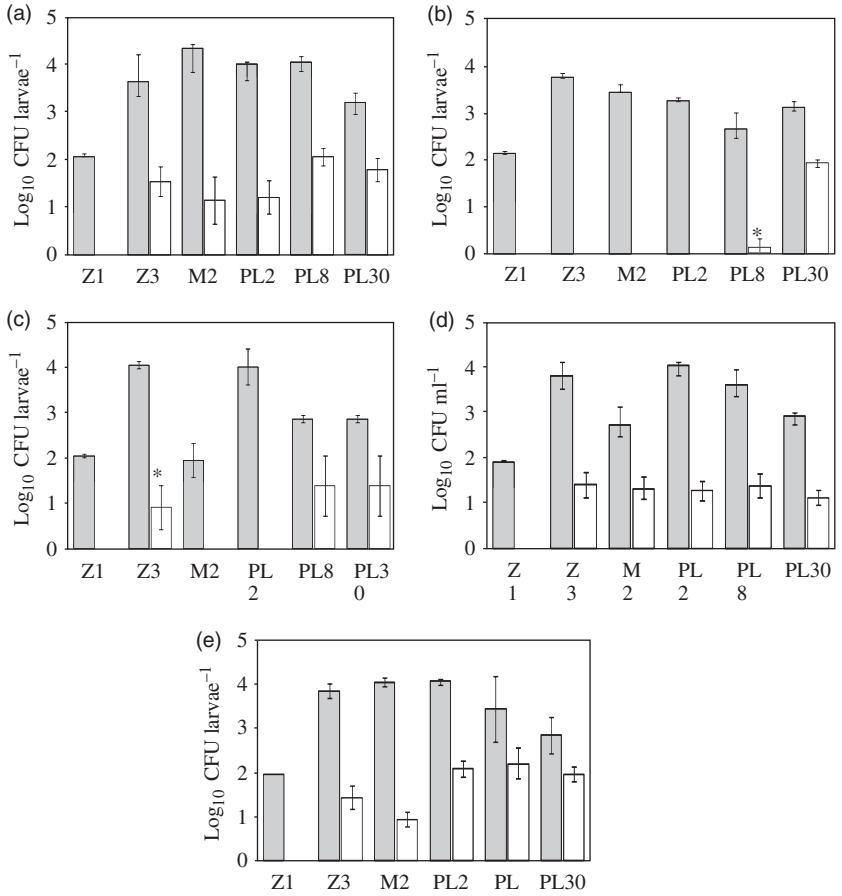

Figure 5 Progression of total heterotrophic bacterial (grey bars) and vibrio population (white bars) in the larvae of *Penaeus monodon* with and without probiotic treatment at different stages of growth. (a) *Bacillus* MCCB 101, (b) *Pseudomonas* MCCB 102, (c) *Pseudomonas* MCCB 103, (d) *Arthrobacter* MCCB 104, (e) control (no treatment); Z1, zoea 1; Z3, zoea 3; M2, mysis 2; PL2, postlarva 2; PL8, postlarva 8; PL30, postlarva 30; *values significant at $P < 0.05$.

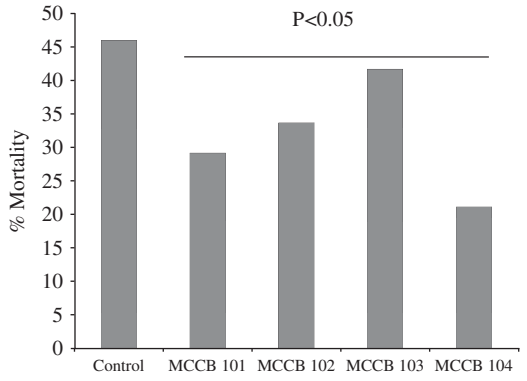

Figure 6 Cumulative mortality (%) of *Penaeus monodon* larvae ($n = 2000$) under treatment with various putative probionts for 38 days.

method to control vibriosis in hatcheries, provided the minimum inhibitory concentration of an antagonistic compound in the culture supernatant is maintained.

Interestingly, although we did not expect a significant change in cumulative mortality in larvae reared with *Bacillus* MCCB 101 and *Arthrobacter* MCCB 104, it was the lowest in these groups in the *in situ* experiment. Based on the *in vitro* experiments, it could be established that this effect was not due to antagonism or competitive inhibition of pathogens. Strains belonging to *Bacillus* spp. were among the first bacterial species to be identified as probiotics for shrimp culture and have been reported to mediate their protection from pathogens through antagonism and/or immunostimulation of the host animals (Moriarty 1998; Rengpipat, Tunyanun, Fast, Piyatiratitivorakul & Menasveta 2003; Hong, Duc & Cutting 2005; Guo *et al.* 2009). Additionally, *Bacillus* spp. could increase the growth and survival of *Fenneropenaeus indicus* by supplementing the digestive system of the host with microbial enzymes and thereby increasing the nutrient utilization (Ziaei-Nejad, Rezaei, Takami, Lovett,

Table 3 Larval health assessment by microscopic examination of postlarvae (PL30) of probiotic-treated and control tanks

	Larval health scores			
Treatment	Size (cm) (% CV)	Colour	Colour and condition of the hepatopancreas, gut condition, fouling, deformity	Muscle:gut ratio (% CV)
Control	2.3 (4.18)	Clear	10	88.33 (0.46)
MCCB101	2.1 (1.92)	Clear	10	88.28 (0.57)
MCCB 102	2.1 (2.05)	Clear	10	87.10 (0.59)
MCCB103	2.2 (2.55)	Clear	10	87.34 (0.60)
MCCB104	2.0 (2.16)	Clear	10	84.58 (0.82)

CV, coefficient of variation.

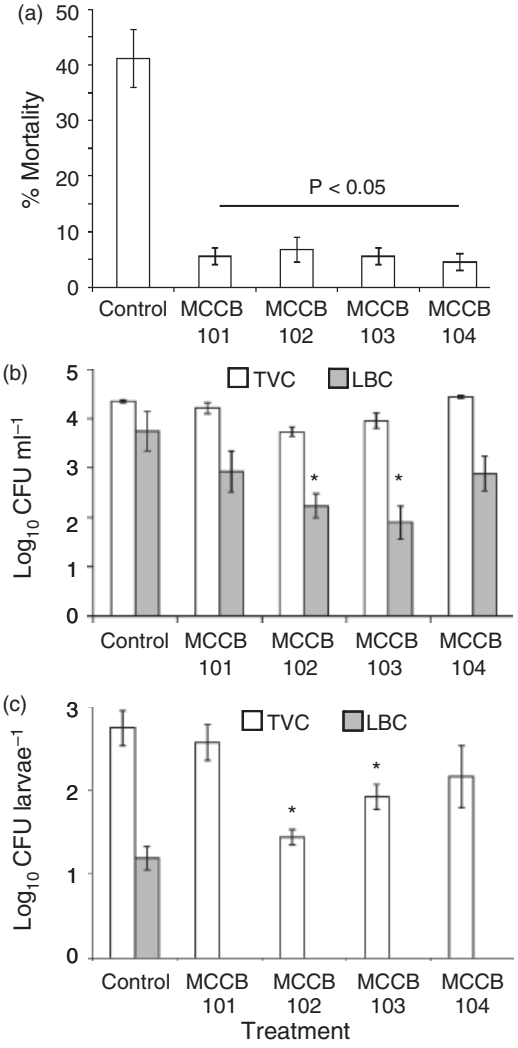

Figure 7 Percentage mortality of *Penaeus monodon* postlarvae, reared with and without the putative probionts following a challenge with *Vibrio harveyi* MCCB 111 (a); total vibrio population (unshaded bars) and luminescent bacterial population (shaded bars) in rearing water (b) and larvae (c); total vibrio population and total luminescent bacterial population; *values significant at $P < 0.05$.

Mirvaghefi & Shakouri 2006). On the other hand, *Arthrobacter* XE-7 did not significantly improve the survival of *P. chinensis* postlarva in mono-treatments but did so only in the presence of *V. parahaemolyticus*, *V. anguillarum* and *V. neries* (Li, Tan, Mai, Ai, Zhang, Xu, Liufu & Ma 2006). In the present study, *Arthrobacter* MCCB 104 significantly improved larval survivals in mono-treatments as well as in the presence of *V. harveyi*. Consequently, the increased survival rate observed with the application of *Bacillus* MCCB 101 and *Arthrobacter* MCCB 104 could be due to an immunostimulatory effect and/or a digestive system-supporting effect because both the strains did not negatively affect the growth of *V. harveyi* MCCB 111. An extension of the current work on the effect of *Bacillus* MCCB 101 and *Arthrobacter* MCCB 104 on the expression of immune genes and the digestive enzyme profile of the target animal would provide comprehensive information to prove the immunostimulatory and digestive enzyme-supporting properties of *Bacillus* MCCB 101 and *Arthrobacter* MCCB 104.

The protection conferred by the probionts was confirmed by challenging the larvae (PL30) with the luminescent bacterium *V. harveyi* MCCB 111. Notably, mortality was significantly lower ($P < 0.05$) in all the probiotic treatments. A high percentage of mortality in the control animals (without probiotic treatment) confirms the pathogenicity of *V. harveyi* MCCB 111. Although the TVC was not significantly different in the rearing water between various treatment groups, it was once again markedly lower in the larvae treated with *Pseudomonas*. Importantly, luminescent bacteria were significantly lower in the rearing water and larvae of the *Pseudomonas* MCCB 102 and 103 treatment groups, confirming their antagonistic property. The absence of luminescent bacteria in the larvae of the groups that received *Bacillus* MCCB 101 and *Arthrobacter* MCCB 104 further points towards an immunostimulatory mode of action by these bacteria.

In conclusion, the present study, along with our earlier reports (Jayaprakash *et al.* 2005; Vijayan *et al.* 2006; Preetha *et al.* 2007), demonstrates that the *Pseudomonas* MCCB 102 and MCCB 103 suppress the *vibrio* population in a *P. monodon* larval rearing system through an antagonistic mode of action. The primary requirement for the demonstration of antagonistic mode of action of probiotics in aquaculture systems is the assurance of the minimum inhibitory concentration of the antagonistic compound in the culture, which is supplemented to the larval rearing tank. Also, this study points towards other possible modes of action such as immunostimulation and enhanced food acceptance leading to improved general health of target animal by probiotics, as evident in the case of *Bacillus* MCCB 101 and *Arthrobacter* MCCB 104. However, further studies at the molecular level are required to elucidate the role of MCCB 101 and MCCB 104 in modulating the expression of immune genes and improving the general health of *P. monodon*.

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Supporting Information

Additional supporting information may be found in the online version of this article:

Table S1: Range of physico-chemical parameters observed in the probiotic treated and untreated rearing water of *Peaneus monodon* during the experiment.
Table S2: Survival of *Penaeus monodon* post larvae (PL-30) after formalin (100 pm) stress test.

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