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Probiotic effect of *Bacillus* NL110 and *Vibrio* NE17 on the survival, growth performance and immune response of *Macrobrachium rosenbergii* (de Man)

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Abstract

Eight hundred and eighty-five strains of bacterial isolates from various samples associated with the natural habitat of *Macrobrachium rosenbergii* were screened for their probiotic potential. Two putative probionts namely *Bacillus* NL110 and *Vibrio* NE17 isolated from the larvae and egg samples, respectively, were selected for experimental studies and were introduced to the juveniles of *M. rosenbergii* (0.080 ± 0.001 g) through different modes such as through feed, water and both. The probiotic potential of the above bacteria in terms of improvements in water quality, growth, survival, specific growth rate (SGR), feed conversion ratio and immune parameters was evaluated. The treatment groups showed a significant improvement in SGR and weight gain ($P < 0.001$). Survival among different treatment groups was better than that in the control group. There were also significant improvements in the water quality parameters such as the concentration of nitrate and ammonia in the treatment groups ($P < 0.05$). Improvements in immune parameters such as the total haemocyte count ($P < 0.05$), phenoloxidase activity and respiratory burst were also significant ($P < 0.001$). It is concluded that screening of the natural microflora of cultured fish and shellfish for putative probionts might yield probiotic strains of bacteria that could be utilized for an environment-friendly and organic mode of aquaculture.

Keywords: *Bacillus*, *Vibrio*, *M. rosenbergii*, probiotics, immune enhancement, water quality

Introduction

During the past three decades, aquaculture has become the fastest-growing food-producing sector and is contributing significantly to national economic development, global food supply and food security. Freshwater prawns such as *Macrobrachium rosenbergii*, which is a preferred species due to several biological characteristics, can be produced in inland locations in closer proximity to large urban markets. *M. rosenbergii* also appears to be resistant to most of the viral diseases that have impacted marine shrimp (Wang, Lo, Chang & Kou 1998). Bacterial diseases are considered to be a menace in the intensive larval culture and production of aquaculture species owing to the large-scale mortality they cause in hatcheries and culture systems (Singh 1990; Grisez & Ollevier 1995; Cheng & Chen 1998). Although antibiotics have played a major role in combating many diseases of cultured aquatic organisms, their indiscriminate use in aquaculture has led to undesirable consequences such as development of antibiotic-resistant bacteria and persistence of antibiotic residues in farm-raised shrimp, etc. (Chaithanya, Nayak & Venugopal 1999).

Recently, the use of probiotics has become a popular and an environment-friendly alternative to antibiotics to improve and maintain a healthy environment for aquaculture. Because shrimp and prawn possess a non-specific immune response, probiotic treatments may provide a broader spectrum and greater non-specific disease protection through competitive exclusion and immune modulation (Rengpipat, Rukpratanporn, Piyatiratitivorakul &

Menasaveta 2000). Several genera of bacteria such as *Bacillus*, *Lactobacillus*,*Vibrio*, *Streptococcus*, *Alteromonas*, *Aeromonas* and *Nitrosomonas* have been used in the aquaculture practices (Gatesoupe 1994; Rengpipat *et al.* 2000; Venkat, Sahu & Jain 2004; Balcazar, Rojas-Luna & Cunningham 2007; Aly, Abd-El-Rahman, John & Mohamed 2008).

Bacillus and *Vibrio* sp. are used widely as probiotics in freshwater aquaculture for improvements in survival, growth and development (Ringo & Vadstein 1998; Huys, Dhert, Robles, Ollevier, Sorgeloos & Swings 2001; Kumar, Mukherjee, Prasad & Pal 2006; Keysami, Saad, Sijam, Daud & Alimon 2007; Apun-Molina, Miranda, Gonzalez, Martinez-Diaz & Rojas-Contreras 2009). They are also used for enhancement in inhibition of pathogenic bacteria (Austin, Stuckey, Robertson, Effendi & Griffith 1995; Gatesoupe 1997; Huys *et al.* 2001), enhancing enzyme activity (Kumar *et al.* 2006; Ghosh, Sinha & Sahu 2008), better feed conversion, fecundity and fry production (Wang & Xu 2006; Ghosh, Sinha & Sahu 2007) as well as for immunity enhancement and disease resistance (Irianto & Austin 2002; Aly, Mohamed & John 2008). However, relatively less work has been directed towards the selection and development of probiotic bacteria for the culture of *M. rosenbergii*. Therefore, the aim of the present research is to screen putative probiotics from the native microflora associated with *M. rosenbergii* and to evaluate their probiotic potential. Two bacterial strains such as *Bacillus* NL110 and *Vibrio* NE17 were evaluated in terms of their antibacterial activity to pathogens in a culture system, pathogenicity to the postlarvae (PL), ability to improve water quality parameters and to enhance the growth, immunity and survival of the juveniles of giant freshwater prawn *M. rosenbergii*.

Materials and methods

Bacterial isolates used for probiotic screening

Four samples each of water, sediment, intestine of *M. rosenbergii*, eggs, larvae, PL and feed samples were analysed for isolation of bacteria. A total of 885 isolates of bacteria, which included 131 isolates from water, 114 from sediment, 155 from intestine of *M. rosenbergii*, 133 from eggs, 206 isolates from larvae and postlarvae of *M. rosenbergii* and 146 isolates from feed samples, were characterized to the genus level and evaluated for probiotic potential. All the samples were collected from Vembanad lake, an important Ramsar site in India and the home ground of *M. rosenbergii*. The isolates were characterized up to the generic level using the taxonomic key by Muroga, Higashi and Keetoku (1987); Barrow and Feltham (1993) and Holt, Krieg, Sneath, Staley and Willliams (2000).

Selection of probiotic bacterial strains

All the 885 bacterial isolates were subjected to preliminary screening for the selection of putative probiotic strains, based on their antibacterial activity against fish, prawn and human pathogens. The source and details of the bacterial pathogens used are given below.

Bacterial pathogens used

In order to determine the antibacterial activity of the probiotic strains, various fish, prawn and human pathogens were used. Fish pathogen *Aeromonas hydrophila* (MTCC 646) and prawn pathogens such as *Vibrio parahaemolyticus* (MTCC 451) and *Vibrio vulnificus* (MTCC 146) were obtained from the culture collection of Institute of Microbial Technology, Chandigarh, India. *Vibrio harveyi* (prawn pathogen) was obtained from the culture collection of School of Environmental Studies, Cochin University of Science and Technology, India. Human pathogens such as *Escherichia coli*, *Salmonella newport* and *Salmonella typhi* were obtained from the culture collection maintained at the School of Environmental Sciences, Mahatma Gandhi University, India.

Antibacterial activity of isolates using the well diffusion and cross-streak method

In order to determine the antibacterial activity of the probiotic strains using the well diffusion method (Chythanya, Karunasagar & Karunasagar 2002), lawn cultures of fish, prawn and human pathogens such as *A. hydrophila*, *V. parahaemolyticus*, *V. harveyi*, *V. vulnificus*, *E. coli*, *S. newport* and *S. typhi* were prepared by pouring 2 mL of a 16–18-h-old tryptic soya broth (TSB) culture of the above microorganisms over sterile tryptic soya agar (TSA) plates. Excess liquid was drained off and the plates were air dried in an incubator (30 °C) for 15 min. Using a sterile gel, puncher wells (3 mm diameter) were punched into the plates. Thirty microlitres of an 18 h culture of the bacterial isolates (885 numbers) in TSB was pipetted into the wells and plates were incubated for 24 h at 30 °C. The presence of antibacterial activity was noted as a clear zone around the wells.

Only those isolates that showed antibacterial activity to the selected fish, prawn and human pathogens in the well diffusion method were selected for the study of antibacterial activity using the cross-streak method (Chythanya *et al.* 2002). This included four bacterial isolates namely *Bacillus* NL110, *Vibrio* NE17, *Aeromonas* NE2 and *Vibrio* NL40. For the detection of antibacterial activity by a cross-streak assay, an 18 h culture of the above bacterial strains was streaked as a 2 cm thick band across the diameter of the TSA plate. After incubation at 30 °C for 24 h, the growth was scraped with a sterile slide. The remaining bacteria were killed by exposure to 5 mL chloroform poured on the glass lid and left for 15 min by keeping the medium inverted over the lid. The plates were then air dried for about 10 min to remove any residual chloroform, and *A. hydrophila*, *V. parahaemolyticus*, *V. harveyi*, *V. vulnificus*, *E. coli*, *S. newport* and *S. typhi* were streaked perpendicular to the bacterial strain band using a sterile glass rod dipped in an 18-h-old culture. The plates were incubated at 30 °C for 24 h. The presence of antibacterial activity was noted as a linear clear zone. Two isolates namely *Bacillus* NL110 and *Vibrio* NE17, which showed good antibacterial activity against the tested pathogens in both the methods, were selected for further screening.

Pathogenicity of probiotic strains to PL of *M. rosenbergii*

Broth cultures (18 h old) of selected probiotic strains (*Bacillus* NL110 and *Vibrio* NE17) were prepared in TSB. The cells were harvested by centrifugation at 1509 *g* for 15 min, washed in physiological saline and the cells were resuspended in 10 mL sterile physiological saline. The cell numbers in the suspension were determined using the spread plate method by plating serial 10-fold dilution in TSA. From 8.35×10^8 cells mL^{-1} of *Bacillus* NL110 and 7.90×10^8 cells mL^{-1} *Vibrio* NE17 in Physiological saline, 0.1 mL was added individually at the beginning of the experiment to each of the 1 L beaker containing 500 mL filter-sterilized (0.45 μm) freshwater to obtain 10^5 cells mL^{-1} of potential probiotic bacteria (Chythanya *et al.* 2002; Ravi, Musthafa, Jegathammbal, Kathiresan & Pandian 2007). To each beaker, 25 healthy PL of *M. rosenbergii* (PL-20, cleared WSSV negative by polymerase chain reaction and showing normal healthy behaviour) collected from a private-owned hatchery, Rosen Fisheries, Kerala, India, were introduced. The pathogenicity of the probiotic strains to PL of *M. rosenbergii* were evaluated by visual observation, swimming activity as well as microscopically for any signs of infection or mortality up to 96 h. The experiment was performed in duplicate with each bacterial strain, and the control was maintained without any bacterial inoculums.

Influence of pH, temperature and salinity on the antibacterial activity of probiotic strains

To determine the effect of pH on antibacterial activity, sterile TSB adjusted to pH 5, 6, 7 and 8 was inoculated with 0.1 mL of an 18-h-old culture of selected probiotic strains (*Bacillus* NL110 and *Vibrio* NE17) and incubated for 24 h at 30 °C. Similarly, the effects of temperature on antibacterial activity were determined at various temperatures such as 10, 20, 30 and 37 °C, and the effect of salinity on antibacterial activity was determined at salinities such as 10, 15, 20, 30 and 35 g L^{-1}. After incubation, the probiotic potential of the bacterial strains was tested against *A. hydrophila*, *V. parahaemolyticus*, *V. harveyi* and *V. vulnificus* using the well diffusion method.

Experimental set-up

The PL (PL-30) of *M. rosenbergii* were grown up to 0.080 ± 0.001 g in a rectangular fibre-reinforced plastic (FRP) tank of 1000 L capacity, supplemented with artificial aeration. Fifteen juveniles of uniform size were placed in a 50 L plastic tank containing 30 L water and were acclimatized for 1 week before the trials began. The juveniles were fed twice daily at 10% body weight per day for a period of 60 days. Waste removal was carried out by exchanging water (2 L) from every tank once in a week. Nine experimental groups with probiotics and a control, all in duplicate, were maintained (Table 1). The control group was fed with normal feed, which was free of any probiotic bacterial inoculation.

Preparation and addition of probiotics

Broth cultures of *Bacillus* NL110 and *Vibrio* NE17 were prepared in TSB and *Lactobacillus plantarum* (MTCC 1325) in Lactobacillus MRS broth by inoculating the isolates into respective broths and incubating at room temperature for 24 h. For the addition of probiotics through water, 5 mL broth cultures of *Bacillus* NL110 ($6.90 \pm 0.34 \times 10^9$ cells mL^{-1}) were directly

Table 1 Details of the experimental groups showing the type of treatment, mode, dose and period of application

Groups	Type of treatment	Mode of application	Dose and period of application
T1	*Bacillus* NL110 through feed	Through feed	$4.73 \pm 2.87 \times 10^9$ CFU g^{-1} fed twice in a day
T2	*Bacillus* NL110 through water	Through water	$1.15 \pm 0.56 \times 10^6$ CFU mL^{-1} once in a week
T3	*Bacillus* NL110 through feed and water	Through feed and water	$4.73 \pm 2.87 \times 10^9$ CFU g^{-1} fed twice in a day $1.15 \pm 0.56 \times 10^6$ CFU mL^{-1} once in a week
T4	*Vibrio* NE17 through feed	Through feed	$4.34 \pm 1.22 \times 10^9$ CFU g^{-1} fed twice in a day
T5	*Vibrio* NE17 through water	Through water	$1.25 \pm 1.23 \times 10^6$ CFU mL^{-1} once in a week
T6	*Vibrio* NE17 through feed and water	Through feed and water	$4.34 \pm 1.22 \times 10^9$ CFU g^{-1} fed twice in a day $1.25 \pm 1.23 \times 10^6$ CFU mL^{-1} once in a week
T7	*Lactobacillus plantarum* through feed	Through feed	$3.16 \pm 2.35 \times 10^9$ CFU g^{-1} fed twice in a day
T8	*L. plantarum* through water	Through water	$1.24 \pm 0.93 \times 10^6$ CFU mL^{-1} once in a week
T9	*L. plantarum* through feed and water	Through feed and water	$3.16 \pm 2.35 \times 10^9$ CFU g^{-1} fed twice in a day $1.24 \pm 0.93 \times 10^6$ CFU mL^{-1} once in a week
Control	Without probiotic application	–	–

CFU, colony-forming unit.

inoculated to Tanks T2 & T3, *Vibrio* NE17 ($7.52 \pm 0.74 \times 10^9$ cells mL^{-1}) to tanks T5 & T6 and *L. plantarum* ($7.41 \pm 0.54 \times 10^9$ cells mL^{-1}) to tanks T8 & T9 once in a week to obtain an approximate concentration of the probiotic bacteria up to 10^6 cells mL^{-1} of water in the concerned experimental tank.

For preparing the probiotic incorporated feed, the cells of *Bacillus* NL110, *Vibrio* NE17 and *L. Plantarum*, after a 24-h incubation, were harvested by centrifugation at 1509 *g* for 15 min. The cells were washed thrice and resuspended in physiological saline. The cells were thoroughly mixed with the commercial scampi feed (Higashimaru Feeds, Aleppy, India) to obtain 10^9 probiotic bacteria g^{-1} in feed (Suralikar & Sahu 2001; Nejad, Rezaei, Takami, Lovett, Mirvaghefi & Shakouri 2006), and the mixture was spread out and aseptically dried in an oven for 1–2 h at 50°C. The bacterial count was checked periodically at a 15-day interval using the spread plate method, and the count was $4.73 \pm 2.87 \times 10^9$ colony-forming unit (CFU) g^{-1} in the *Bacillus* NL110-incorpo-rated feed, $4.34 \pm 1.22 \times 10^9$ CFU g^{-1} in the *Vibrio* NE17-incorporated feed, $3.16 \pm 2.35 \times 10^9$ CFU g^{-1} in the *L. plantarum*-incorporated feed and $5.01 \pm 1.78 \times 10^5$ CFU g^{-1} in the normal feed. Feed was stored in clean, plastic bottles at 4°C and provided to the respective experimental tanks. Probiotic-incorporated feeds were prepared twice weekly.

Physico-chemical parameters

Water samples were collected from the experimental tanks on a weekly basis and analysed for changes in the water quality parameters such as pH, temperature, dissolved oxygen (DO), ammonia and nitrite. The pH of the water sample was analysed using an electronic pH meter (Systronics, μ pH System 361, Chennai, India), temperature using a temperature probe (Systronics, μ pH System 361), DO using the Winkler method, ammonia using the phenol–hypochlorite method and nitrite using the colorimetric method as per the American Public Health Association (APHA 1998).

Bacteriological analysis

Preparation of samples

For bacteriological analysis, water samples from the experimental tanks were collected at a 15-day interval and were serially diluted aseptically up to 10^{-4} using sterile-distilled water. For the analysis of the intestinal microflora of juveniles in the initial stage, the specimens were surface washed in a sterile 0.1% benzalconium chloride solution and then thoroughly rinsed in sterile-distilled water. The samples were then filtered using a sterile filter paper and excess water was removed using a sterile blotting paper. The PL samples were then homogenized in a sterile all-glass homogenizer and serially diluted up to 10^{-5}. For analysis of the intestinal microflora of juveniles of *M. rosenbergii* at the final stage, the intestine was removed aseptically, homogenized in a sterile all-glass homogenizer and serially diluted up to 10^{-5}. For the analysis of feed samples, the feed samples were aseptically weighed and homogenized in a sterile all-glass homogenizer and serially diluted up to 10^{-7}.

Estimation of the total viable count

Aliquots of 0.2 mL samples from each dilution of water samples, intestinal samples and feed samples were spread plated in triplicate on TSA for the enumeration of the total aerobic heterotrophic bacteria, expressed as total viable count (TVC). The plates were then incubated at 30 °C for 24–48 h. After incubation, plates with 30–300 CFU were selected for counting and expression of TVC of bacteria.

Growth performance of juveniles

Juveniles were weighed initially and at the end of the experiment to assess the growth performance in terms of specific growth rate (SGR) and the feed conversion ratio (FCR). SGR and FCR were calculated using the following formula:

$$\text{SGR} = \frac{ln\,\text{final body wt} - ln\,\text{initial body wt}}{60\,\text{days}} \times 100$$

where, ln = natural log

$$\text{FCR} = \frac{\text{Quantity of feed consumed}}{\text{Total weight gain}}$$

Immunological parameters of the treated juveniles

Total haemocyte count (THC)

Haemolymph (HL) from each prawn was collected from the rostral sinus cavity using a 26 G needle and a 1 mL syringe containing an anticoagulant. HL was collected from two randomly selected juveniles from each treatment group at 5 p.m. Haemocytes were counted using a haemocytometer and expressed as cells mL^{-1} of HL.

Phenoloxidase (PO) activity

Phenoloxidase activity was estimated spectrophotometrically using L-3, 4-dihydroxyphenyl-alanine (L-DOPA) as a substrate (Soderhall 1981). HL in an anticoagulant (100 μL) was incubated for 30 min at room temperature after adding 100 μL of sodium dodecyl sulphate (SDS) in a small cuvette. After the incubation, 1000 μL of enzyme substrate L-DOPA was added and the optic density was measured at 490 nm. Absorbance measurements were performed against a blank consisting of SDS and L-DOPA. One unit of enzyme activity was defined as an increase in absorbance min^{-1} 100 μL HL^{-1}.

Respiratory burst

The respiratory burst or super oxide generation (O_2^-) of haemocytes was quantified using a nitroblue tetrazolium (NBT) assay (Song & Hsieh 1994). HL in the anticoagulant (100 μL) was incubated for 30 min at 10°C after adding 100 μL of NBT salt solution. After incubation, the mixture was centrifuged at 1610 *g* for 10 min. After removing the supernatant solution, the cells were washed with phosphate-buffered saline. Then, 100 μL of 100% methanol was added and incubated at room temperature for 10 min. The solution was then centrifuged again at 3622 *g* for 10 min. The supernatant was removed and the tubes were air dried for 30 min at room temperature. The pellet was then rinsed with 50% methanol three to four times and coated with 120 μL KOH (2 M) and 140 μL dimethyl sulphoxide (DMSO) solution to dissolve the formazan. The optical absorbances of the mixtures were read at 620 nm. The results were expressed as an increase in absorbance 100 μL HL^{-1}.

Statistical analysis

The data were analysed by two-factor analysis of variance (ANOVA) using the statistical tool package of MICROSOFT OFFICE EXCEL 2007 software. Wherever the treatments were found to be significant, least significance was calculated and significant treatments were identified. Whenever necessary, the results are presented as the average and standard deviation.

Results and Discussion

Selection of bacterial isolates with probiotic potential

From a total of 885 bacterial isolates from a natural environment and different life stages of *M. rosenbergii*, four were selected initially based on their antibacterial activity against fish, prawn and human pathogens. The pathogenicity of the selected pathogens was well documented by several researchers. Even though *Aeromonas* spp. are considered to be opportunistic pathogen in the hatchery and culture environment of *M. rosenbergii* (New 1995), they have been linked to outbreaks of disease like burn spot disease (El-Gamal, Alderman, Rodgers, Polglase & Macintosh 1986), black-spot, bacterial necrosis and gill obstruction in larvae (Lombardi & Labao 1991a, b) and septicaemia under adverse conditions (Sung, Hwang & Tasi 2000). *Vibrio* sp. including

V. parahaemolyticus, *V. harveyi* and *V. vulnificus* are the most important bacterial pathogens of cultured shrimp and prawn responsible for a number of diseases and mortalities (Lightner 1983; Chen, Hanna, Altman, Smith, Moon & Hammond 1992; Lavilla-Pitogo, Leano & Paner 1998; Martin, Rubin & Swanson 2004; Khuntia, Das, Samantaray, Samal & Mishra 2008). The ability of human pathogens such as *E. coli* and *Salmonella* to cause gastrointestinal symptoms such as fever, diarrhoea, abdominal pain, nausea and vomiting in human is also well documented (Bhan, Bahl & Bhatnagar 2005; Ministry of Health & Welfare 2006; Hamner, Broadway, Mishra, Tripathi, Mishra, Pulcini, Pyle & Ford 2007; Chugh 2008; Woc-Colburn & Bobak 2009).

The results of the antibacterial activity of the bacterial strains isolated from the natural environment of *M. rosenbergii* against the pathogenic bacteria (Table 2) revealed that the two bacterial strains, *Bacillus* NL110 isolated from larvae and *Vibrio* NE17 from egg of *M. rosenbergii*, had better probiotic potential than *Aeromonas* NE2 from the eggs and *Vibrio* NL40 from the larvae of *M. rosenbergii*. *Bacillus* NL110 showed antibacterial activity against all the tested fish and prawn pathogens, while *Vibrio* NE17 showed antibacterial activity against 3 of them. Our observations on the antibacterial activity of *Bacillus* to pathogens of fish and prawn were similar to those previously reported by Balcazar and Rojas-Luna (2007). Aquatic candidate probionts for larviculture isolated from adults (Rengpipat *et al.* 2000; Riquelme, Araya & Escribano 2000; Gullian, Thompson & Rodriguez 2004) and healthy larvae (Gatesoupe 1997; Ringo & Vadstein 1998) have been reported previously. It has been suggested that the efficacy of probiotics is likely to be highest in the host species from where they were isolated (Verschuere, Rombaut, Sorgeloos & Verstraete 2000). It is also reported that the non-pathogenic strains of known pathogenic bacteria like *V. alginolyticus*, *A. media* and *A. hydrophila* also showed antibacterial activity and have been used as a probiotic in algal production, shrimp culture, oyster culture and fish culture (Austin *et al.* 1995; Gibson, Woodworth & George 1998; Gomez-Gil, Roque & Velasco-Blanco 2002; Irianto & Austin 2002). It is possible that when pathogenicity is suppressed or lost, other factors such as growth rate or attachment ability, which are factors that contribute to their success as pathogens, may influence the microflora to the benefit of its host.

Pathogenicity of probiotic strains to *M. rosenbergii* PL

Figure 1 shows the pathogenicity effect of *Bacillus* NL110 and *Vibrio* NE17 on *M. rosenbergii* PL. The results showed that *Bacillus* NL110 and *Vibrio* NE17 have no pathogenic effect on the PL of *M. rosenbergii*. The survival of PL was higher in both experimental groups when compared with the control ($P < 0.01$). One of the most important criteria for a candidate to be used in biocontrol is that the organism should be non pathogenic to the host. Because the probiotic strain *Bacillus* NL110 was isolated from *M. rosenbergii* larvae and *Vibrio* NE17 from the eggs collected from the natural habitat, these bacterial strains might have already been selected by the host larvae because of their positive effects on them. Similar findings on probiotic strains were reported earlier (Chythanya *et al.* 2002; Vijayan, Bright Singh, Jayaprakash, Alavandi, Somnath Pai, Preetha, Rajan & Santiago 2006).

Table 2 Results of antibacterial activity by potential probiotic bacteria against different pathogenic bacteria

	Antibacterial activity of potential probiotic strains							
	***Aeromonas* NE2**		***Vibrio* NE17**		***Vibrio* NL40**		***Bacillus* NL110**	
Pathogens tested	**WDM**	**CSM**	**WDM**	**CSM**	**WDM**	**CSM**	**WDM**	**CSM**
Aeromonas hydrophila	X	X	X	X	X	X	X	X
Vibrio parahaemolyticus	X	X	X	X	X	X	X	X
Vibrio harveyi	–	–	–	–	–	–	X	X
Vibrio vulnificus	–	–	X	X	–	–	X	X
Escherichia coli	X	X	X	X	–	–	X	X
Salmonella newport	–	–	X	X	X	X	–	–
Salmonella typhi	–	–	–	–	–	–	X	X

WDM, well diffusion method; CSM, cross-streak method, X, positive for antibacterial activity.

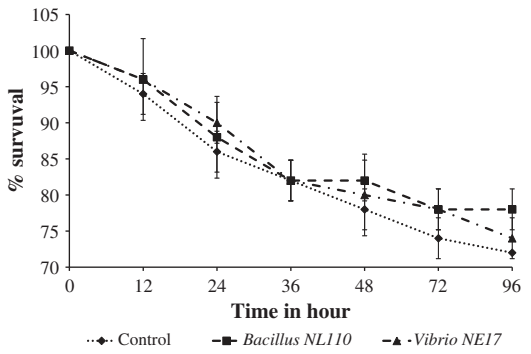

Figure 1 Effects of *Bacillus* NL110 and *Vibrio* NE17 on the survival of PL of *Macrobrachium rosenbergii*.

Influence of growth conditions on the antibacterial activity of probiotic strains

The growth and antibacterial activities of probiotic strains *Bacillus* NL110 and *Vibrio* NE17 against 4 pathogenic bacteria at different pH, temperature and salinities are presented in Table 3. Bacterial isolates did not show growth at pH 5 and at 10 °C. However, the selected strains did not show antibacterial activity against *V. harveyi* and *V. vulnificus* at pH 6. At pH 7 and 8, *Bacillus* NL110 showed antibacterial activity against all the tested pathogens, while *Vibrio* NE17 showed antibacterial activity only against *A. hydrophila*, *V. parahaemolyticus* and *V. vulnificus*.

Bacillus NL110 showed antibacterial activity against all the 4 pathogens in a wide range of temperatures such as 20, 30 and 37 °C, except against *V. harveyi* at 20 °C. Unlike *Bacillus* NL110, *Vibrio* NE17 exhibited antibacterial activity against *A. hydrophila*, *V. parahaemolyticus* and *V. vulnificus* only at 30 and 37 °C. *Bacillus* NL110 and *Vibrio* NE17 showed good growth and antibacterial activity at all the salinities tested in the present study. Because the probiotic strains have to be used in the field under different environmental conditions, the ability of the strains to grow over a wide salinity and temperature range has huge significance. The study revealed that both the probiotic strains can be used in freshwater as well as in marine aquaculture under a wide temperature and salinity range. The range of environmental conditions over which the organisms in the present study could grow and show activity was wider than those reported by Chythanya *et al.* (2002), who studied the production of the antibacterial component of the *Pseudomonas* I-2 strain under different environmental conditions. Our findings were also supported by the observations of Jayaprakash, Pai, Anas, Preetha, Philip and Singh (2005) and Vijayan *et al.*

Table 3 Growth and antibacterial activity of *Bacillus* NL110 and *Vibrio* NE17 at different pH, temperatures and salinities

Potential probiotic strains	pH	Growth	Antibacterial activity against				Temperature (°C)	Growth	Antibacterial activity against				Salinity (mg L^{-1})	Growth	Antibacterial activity against			
			A. hydrophila	*V. parahaemolyticus*	*V. harveyi*	*V. vulnificus*			*A. hydrophila*	*V. parahaemolyticus*	*V. harveyi*	*V. vulnificus*			*A. hydrophila*	*V. parahaemolyticus*	*V. harveyi*	*V. vulnificus*
Bacillus	5	–	–	–	–	–	10	–	–	–	–	–	10	+	X	X	X	X
NL110	6	+	X	X	–	X	20	+	X	X	–	X	15	+	X	X	X	X
	7	+	X	X	X	X	30	+	X	X	X	X	20	+	X	X	X	X
	8	+	X	X	X	X	37	+	X	X	X	X	30	+	X	X	X	X
													35	+	X	X	X	X
Vibrio	5	–	–	–	–	–	10	–	–	–	–	–	10	+	X	X	–	X
NE17	6	+	X	X	–	–	20	+	X	X	–	–	15	+	X	X	–	X
	7	+	X	X	–	X	30	+	X	X	–	X	20	+	X	X	–	X
	8	+	X	X	–	X	37	+	X	X	–	X	30	+	X	X	–	X
													35	+	X	X	–	X

X, positive for antibacterial activity; +, indicates growth.
A. hydrophila, *Aeromonas hydrophila*; *V. parahaemolyticus*, *Vibrio parahaemolyticus*; *V. harveyi*, *Vibrio harveyi*; *V. vulnificus*, *Vibrio vulnificus*.

(2006), who suggested the possibility of developing a biological method of suppressing vibrios associated with prawn larval rearing systems.

Physico-chemical parameters of water

As all the treatment and control tanks were maintained at room temperature at controlled aeration; there was no significant variation in the average temperature and DO (29.5–29.8 °C and 6.57–6.84 mg L^{-1}) among the different treatment groups and control. The water pH of all the treatment groups was slightly lower than that of the control (7.78), and the lowest average pH was observed from the T8 group (7.60). The observed physico-chemical parameters of the water such as temperature, DO and pH of the water from the treatment tanks were within the optima for these factors for *M. rosenbergii* culture. Because *M. rosenbergii* could tolerate a wide range of temperature (14–35 °C), pH (7.0–8.5) and salinity levels (0–25 g L^{-1}), the minor variations in the treatment groups did not seem to affect the survival of the organism. The results of the present study were comparable to those of Venkat *et al.* (2004), who had reported on the growth and survival of PL of *M. rosenbergii* with a *Lactobacillus*-based probiotic feed.

The ammonia concentration of the water from the treatment tanks ranged from 0.0125 to 0.0985 mg L^{-1} and the nitrite concentration from 0.0205 to 0.1200 mg L^{-1} during the experimental period, and there were significant differences in the average ammonia (0.042–0.056 mg L^{-1}) and nitrite (0.054–0.075 mg L^{-1}) concentration among different treatments (Fig. 2, $P < 0.05$). The ammonia concentration of water was the lowest in the T4 group, followed by the T1 and T2 groups. Significantly lower values of nitrite were observed in the T5, T6, T2 and T3 groups respectively. The reduction in ammonia and nitrite in the probiotic-treated tanks may be because of the action of probiotic strains *Bacillus* NL110 and *Vibrio* NE17. Bioremediation of water and biocontrol of pathogens (Queiroz & Boyd 1998; Gatesoupe 1999; Skjermo & Vadstein 1999) are the 2 major modes of action of administering beneficial bacteria in the culture water. When commercial microbial products are used to treat culture wastewater, heterotrophic bacterial assimilation could be the main and powerful mechanism for organic matter removal and conversion of potentially toxic inorganic nitrogen into relatively stable organic nitrogen. It has been reported that the use of *Bacillus* spp. improved the water quality, survival and growth rates and increased the health status of juvenile *P. monodon* (Dalmin, Kathiresan & Purushothaman 2001). Decamp, Moriarty and Lavens (2008) reported that improved water quality was as a major factor for better performance in biomass increase and concluded that probiotic bacteria such as *Bacillus* spp. have the potential to induce growth enhancement comparable to that obtained with antimicrobials, and importantly, in a cost-effective manner.

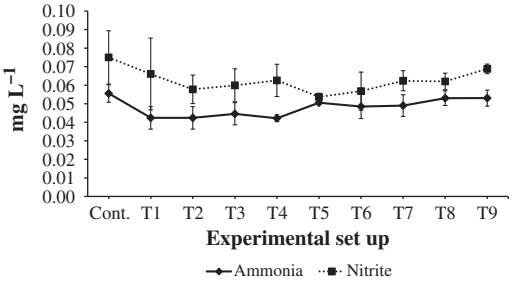

Figure 2 Average ammonia and nitrite values of *Macrobrachium rosenbergii* juvenile rearing water where probiotic strains were introduced through different routes.

TVC from various samples

The initial TVC load of culture water, commercial scampi feed and the intestine of *M. rosenbergii* were $3.54 \pm 0.08 \times 10^3$ CFU mL^{-1}, $4.95 \pm 0.07 \times 10^5$ CFU g^{-1} and $1.26 \pm 0.34 \times 10^3$ CFU Juvenile^{-1} respectively. After incorporating the probiotic, the TVC of culture water was 10^6 CFU mL^{-1} and that of the feed was 10^9 CFU g^{-1}. There were no significant differences between the bacterial counts among the probiotic-incorporated culture water and prawn feed ($P > 0.05$). Thereafter, the TVC load of the rearing water was analysed at a 15-days interval, up to 60 days, in the different experimental groups (Table 4). There were four-log increases in the TVC of the water from the control tank and the rearing tank water where probiotics were introduced through feed. There was an increase of one log in the TVC of the rearing tank water in which probiotics were introduced through water.

There was a significant difference in TVC during the culture period ($P < 0.001$). The lowest TVC levels were observed on the first and the 16th day, while the highest TVC load was found on the 60th day. The fluctuation in the range of the THB load was dependent on the quality of feed, removal of unconsumed feed and the method used in the culture system

Table 4 Total viable count (TVC) of water from different treatment and control groups of *Macrobrachium rosenbergii* juveniles at different time intervals

	TVC mL^{-1} of water at different time intervals				
Treatment groups	**1st day**	**16th day**	**31st day**	**46th day**	**60th day**
T1 (*Bacillus* NL110 through feed)	$3.54 \pm 0.08 \times 10^3$	$1.16 \pm 0.06 \times 10^7$	$2.28 \pm 0.11 \times 10^7$	$4.13 \pm 0.18 \times 10^7$	$4.88 \pm 1.31 \times 10^7$
T2 (*Bacillus* NL110 through water)	$1.45 \pm 0.07 \times 10^6$	$1.20 \pm 0.00 \times 10^7$	$3.01 \pm 1.19 \times 10^7$	$3.94 \pm 0.26 \times 10^7$	$5.45 \pm 1.06 \times 10^7$
T3 (*Bacillus* NL110 through feed and water)	$1.45 \pm 0.07 \times 10^6$	$5.40 \pm 1.56 \times 10^6$	$3.43 \pm 0.72 \times 10^7$	$2.85 \pm 0.96 \times 10^7$	$5.31 \pm 1.83 \times 10^7$
T4 (*Vibrio* NE17 through feed)	$3.54 \pm 0.08 \times 10^3$	$1.14 \pm 0.06 \times 10^7$	$2.93 \pm 0.39 \times 10^7$	$4.73 \pm 0.32 \times 10^7$	$5.40 \pm 1.98 \times 10^7$
T5 (*Vibrio* NE17 through water)	$1.40 \pm 0.07 \times 10^6$	$1.26 \pm 0.34 \times 10^7$	$3.51 \pm 1.90 \times 10^7$	$4.51 \pm 0.72 \times 10^7$	$5.94 \pm 0.37 \times 10^7$
T6 (*Vibrio* NE17 through feed and water)	$1.40 \pm 0.07 \times 10^6$	$6.25 \pm 1.34 \times 10^6$	$4.03 \pm 0.44 \times 10^7$	$2.66 \pm 0.91 \times 10^7$	$5.74 \pm 1.22 \times 10^7$
T7 (*Lactobacillus plantarum* through feed)	$3.54 \pm 0.08 \times 10^3$	$3.34 \pm 3.22 \times 10^7$	$4.20 \pm 0.85 \times 10^7$	$6.90 \pm 2.99 \times 10^7$	$9.18 \pm 0.17 \times 10^7$
T8 (*L. plantarum* through water)	$1.46 \pm 0.13 \times 10^6$	$1.67 \pm 0.76 \times 10^7$	$3.77 \pm 1.06 \times 10^7$	$6.29 \pm 2.46 \times 10^7$	$6.15 \pm 1.91 \times 10^7$
T9 (*L. plantarum* through feed and water)	$1.46 \pm 0.13 \times 10^6$	$2.44 \pm 1.50 \times 10^7$	$1.85 \pm 0.49 \times 10^7$	$3.65 \pm 0.92 \times 10^7$	$7.77 \pm 1.87 \times 10^7$
Control	$3.54 \pm 0.08 \times 10^3$	$1.68 \pm 0.74 \times 10^7$	$1.78 \pm 0.95 \times 10^7$	$3.62 \pm 0.54 \times 10^7$	$6.11 \pm 0.27 \times 10^7$

Table 5 Intestinal TVC load of juveniles of *Macrobrachium rosenbergii* from different experimental groups and control

Treatment groups	**TVC load (CFU J^{-1})**
T1 (*Bacillus* NL110 through feed)	$3.15 \pm 2.47 \times 10^7$
T2 (*Bacillus* NL110 through water)	$2.90 \pm 0.14 \times 10^6$
T3 (*Bacillus* NL110 through feed and water)	$2.85 \pm 0.49 \times 10^7$
T4 (*Vibrio* NE17 through feed)	$4.50 \pm 2.12 \times 10^5$
T5 (*Vibrio* NE17 through water)	$6.90 \pm 0.14 \times 10^5$
T6 (*Vibrio* NE17 through feed and water)	$7.00 \pm 0.71 \times 10^5$
T7 (*Lactobacillus plantarum* through feed)	$5.15 \pm 1.48 \times 10^5$
T8 (*L. plantarum* through water)	$4.65 \pm 2.19 \times 10^5$
T9 (*L. plantarum* through feed and water)	$5.70 \pm 2.40 \times 10^5$
Control	$4.90 \pm 0.85 \times 10^5$

CFU, colony-forming unit.

(Anderson, Shamsudin & Nash 1989). It was reported that the TVC normally found in earthen ponds used for commercial shrimp culture was in the range of 10^7 to 10^8 CFU mL^{-1} (Colorni 1985), which was similar to the TVC levels found in our study. However, Rengpipat, Phianphak, Piyatiratitivorakul and Menasveta (1998) reported a TVC of about 10^{10} cells mL^{-1}, which was higher than that observed in the present study. The above researchers argued that higher TVCs in the culture system were probably due to the low algal density and the lack of grazer organisms.

The TVC load of the intestine of *M. rosenbergii* juveniles was estimated after the completion of the experiment from all the experimental tanks (Table 5). A TVC load of 10^5 CFU J^{-1} was found in most of the experimental groups. While there was a one-log increase in the TVC load of the intestine of *M. rosenbergii* juveniles in the T2 group, it was found to increase by two logs among the juveniles from the T1 and T3 groups. This is an indication of intestinal attachment and successful colonization of probiotic bacteria in the intestine. The TVC loads found in the present study were comparable to the TVC levels in the digestive tract of *M. rosenbergii* from culture ponds (Lalitha & Surendran 2004; Uddin & Al-Harbi 2005). Rengpipat *et al.* (2000) also reported a bacterial count in the range of 10^6–10^8 CFU g^{-1} from shrimp intestine during the probiotic treatment study.

Survival and growth of *M. rosenbergii* juveniles

The survival of *M. rosenbergii* juveniles (Table 6) showed that there was no significant difference among the treatment groups ($P > 0.05$). The results of the weight gain and SGR (Table 6) by *M. rosenbergii* juvenile showed that there was a significant difference among the treatment groups ($P < 0.001$). Among

Table 6 Percentage survival, final weight, SGR and FCR of *Macrobrachium rosenbergii* juveniles from control and different treatment groups with probiotic bacterial strains incorporated through different routes

Treatment groups	Survival (%)	Final weight (g)	SGR	FCR
T1 (*Bacillus* NL110 through feed)	93.3 ± 0.00	0.703 ± 0.004	3.60 ± 0.008	2.17 ± 0.01
T2 (*Bacillus* NL110 through water)	90.0 ± 4.67	0.805 ± 0.007	3.87 ± 0.015	1.97 ± 0.12
T3 (*Bacillus* NL110 through feed and water)	93.3 ± 0.00	0.630 ± 0.014	3.46 ± 0.037	2.42 ± 0.05
T4 (*Vibrio* NE17 through feed)	86.7 ± 0.00	0.754 ± 0.063	3.73 ± 0.139	2.18 ± 0.18
T5 (*Vibrio* NE17 through water)	86.7 ± 0.00	0.763 ± 0.025	3.78 ± 0.054	2.16 ± 0.07
T6 (*Vibrio* NE17 through feed and water)	96.7 ± 4.74	0.820 ± 0.006	3.90 ± 0.013	1.80 ± 0.07
T7 (*Lactobacillus plantarum* through feed)	86.7 ± 0.00	0.739 ± 0.011	3.70 ± 0.024	2.23 ± 0.03
T8 (*L. plantarum* through water)	90.0 ± 4.67	0.780 ± 0.009	3.82 ± 0.020	2.03 ± 0.08
T9 (*L. plantarum* through feed and water)	90.0 ± 4.67	0.756 ± 0.011	3.76 ± 0.025	2.10 ± 0.08
Control	86.7 ± 0.00	0.629 ± 0.030	3.44 ± 0.079	2.62 ± 0.12

SGR, specific growth rate; FCR, feed conversion ratio.

the different treatment groups that showed a positive effect, *M. rosenbergii* juveniles from the T2 and T6 groups showed the highest weight gain, SGR and the lowest FCR. Significant increases in the growth of *P. monodon* and *P. vannamei* with different strains of *Bacillus* spp. and *Vibrio* spp. incorporated into the feed were reported previously (Rengpipat *et al.* 1998; Gullian *et al.* 2004). Garriques and Arevalo (1995) reported better shrimp growth in commercial *P. vannamei* hatcheries, treated with *V. alginolyticus*, while Wang (2007) observed a significantly higher mean weight in probiotic-treated groups of *P. vannamei* than that of the control. Venkat *et al.* (2004) and Nejad *et al.* (2006) reported higher percentage weight gain, SGR and FCR of PL of *M. rosenbergii* and Indian white shrimp using *Lactobacillus* spp. and *Bacillus* respectively. The results of our studies also reconfirm the efficacy of probiotics in increasing the SGR of cultured shrimp.

It was reported by Wang (2007) and Zhou, Wang and Li (2009) that application of probiotics induced digestive enzyme activity and enhanced the survival of *P. vannamei*. Furthermore, bacteria, particularly members of the genus *Bacillus*, secrete a wide range of exo-enzymes (Moriarty 1996, 1998), which might help them compete with bacterial pathogens for nutrients and thereby inhibit the growth of pathogens. Such probiotic strains were suggested as a valid alternative to the prophylactic application of chemicals (Decamp *et al.* 2008). Microorganisms are capable of producing complex molecules, either directly, as part of their metabolic activities, or indirectly, when they die. The compounds that may benefit the host include pigments (Holmstrom, Egan, Franks, McCloy & Kjelleberg 2002), proteins (Klein, Pack, Bonaparte & Reuter 1998), fatty acids (Shirasaka, Nishi & Shimizu 1995), vitamins (Sugita, Miyajima & Deguchi 1991) and digestive enzymes (Cahill 1990; Hansen & Olafsen 1999; Ramirez & Dixon 2003). A problem with many cultured species is the assimilation of artificial diets during the early stages of larval development. It has been suggested that this is due to the low enzyme levels capable of digesting the food (Govoni, Boehlert & Watanabe 1986). Probiotic strains with good exoenzyme potential could help overcome this problem. Nejad *et al.* (2006) observed increases in the specific activities of digestive enzymes in probiotic treatments that led to enhanced digestion and increased absorption of food. Therefore, the addition of probiotic bacteria capable of producing beneficial enzymes may aid in the digestion of artificial foods, thereby reducing the live food feeding period and the subsequent associated costs. However, further studies are needed to determine the exact role of any metabolite produced by the proposed probiotics *Bacillus* NL110 and *Vibrio* NE17.

Immunological parameters of *M. rosenbergii* juveniles treated with probiotics

THC

The THC of *M. rosenbergii* juveniles after the experimental period (Table 7) showed a significantly higher THC than that of the T4 and T6 treatment groups ($P < 0.05$). The increase in the haemocyte count observed among *M. rosenbergii* juveniles in the present study was comparable to the observations of Cheng and Chen (2001) in *M. rosenbergii* and Chand and Sahoo (2006) in *M. malcomsonii*. Rodriguez, Espinosa, Echeverria, Cardenas, Roman and Stern (2007) reported increased THC in *P. vannamei* larvae and

Table 7 Variation in the THC, PO and respiratory burst values of *Macrobrachium rosenbergii* juveniles from control and different treatment groups with probiotic bacterial strains incorporated through different routes

	Immunological parameters		
Treatment groups	**Haemocyte (log_{10} cells mL^{-1})**	**PO (OD min^{-1} 100 μL HL^{-1})**	**Respiratory burst (OD 100 μL HL^{-1})**
T1 (*Bacillus* NL110 through feed)	6.7355 ± 0.039	0.731 ± 0.016	1.872 ± 0.101
T2 (*Bacillus* NL110 through water)	6.7024 ± 0.011	0.612 ± 0.010	1.742 ± 0.012
T3 (*Bacillus* NL110 through feed and water)	6.6787 ± 0.023	0.435 ± 0.021	1.754 ± 0.008
T4 (*Vibrio* NE17 through feed)	7.4272 ± 0.359	0.482 ± 0.028	1.781 ± 0.034
T5 (*Vibrio* NE17 through water)	6.8431 ± 0.089	0.502 ± 0.033	1.772 ± 0.033
T6 (*Vibrio* NE17 through feed and water)	7.1818 ± 0.227	0.494 ± 0.045	1.277 ± 0.103
T7 (*Lactobacillus plantarum* through feed)	6.5812 ± 0.102	0.511 ± 0.006	1.239 ± 0.013
T8 (*L. plantarum* through water)	6.6337 ± 0.048	0.477 ± 0.014	1.464 ± 0.132
T9 (*L. plantarum* through feed and water)	6.9173 ± 0.074	0.544 ± 0.030	1.832 ± 0.229
Control	6.6956 ± 0.192	0.365 ± 0.022	1.320 ± 0.099

THC, total haemocyte count; PO, phenoloxidase.

juveniles after treatment with the probiotic, *V. alginolyticus*. The circulating haemocyte or THC of decapod crustacean plays an important role in regulating the physiological functions including hardening of exoskeleton, wound repair, carbohydrate metabolism, transport and storage of protein and amino acid and HL coagulation (Ratcliffe, Rowley, Fitzgerald & Rhodes 1985; Martin, Hose, Omori, Chong, Hoodbboy & McKrell 1991). Other than the physiological functions, haemocytes play a crucial role in nonspecific cellular immunity against pathogens and parasites, including the primary immune responses of phagocytosis, encapsulation, nodule formation, cytotoxic mediation and the proPO-activating system (Anderson 1992). The enhanced THC among the juveniles of *M. rosenbergii* from the treatment groups further boosts the possible probiotic potential of *Bacillus* NL110 and *Vibrio* NE17.

PO and respiratory burst

The PO activity and respiratory burst study in the HL of *M. rosenbergii* juveniles (Table 7) showed significant differences among the experimental groups ($P < 0.001$). A significantly higher PO was observed in the treatment group T1, followed by T2, while a high respiratory burst was observed in the T1 group, followed by the T9 group. The results of PO obtained correspond to those obtained by Rengpipat *et al.* (2000) in *P. monodon* treated with *Bacillus* S11 strain and in *P. vannamei* stimulated with *Bacillus* P64, *Vibrio* P62 and *V. alginolyticus* (Gullian *et al.* 2004). A recent study of Tseng, Ho, Huang, Cheng, Shiu, Chiu and Liu (2009) reported increased resistance of shrimp through immune modifications such as increases in PO activity, phagocytic activity and clearance efficiency against *V. alginolyticus* after *B. subtilis* E20 consumption. However, the immune values cannot be compared because the techniques used for PO activity are different from those used in the present study. Because *Bacillus* is a long-term resident in probiotic-treated shrimp guts, it should provide a longer-term immunostimulation for shrimp compared with glucan or other such immunostimulants (Sung, Kou & Song 1994). Improvements in respiratory burst after treatment with probiotics such as *V. vulnificus* (Sung, Yang & Song 1996), *L. rhamnosus*, *Enterococcus faecium* and *B. subtilis* (Nikoskelainen, Ouwehand, Bylund, Salminen & Lilius 2003; Panigrahi, Kiron, Satoh, Hirono, Kobayashi, Sugita, Puangkaew & Aoki 2007) were also reported. The generation of superoxide anions plays an important role in microbicidal activity and it has been reported in the haemocytes of the crab *Carcinus maenas* (Bell & Smith 1993), tiger shrimp *P. monodon* (Song & Hsieh 1994) and blue shrimp *P. stylirostris* (Le Moullac, Soyez, Saulnier, Ansquer, Avarre & Levy 1998).

Many different antibacterial compounds are produced by a range of *Bacillus* spp., and it appears that other bacteria would be unlikely to have resistance genes to all of the antibacterials produced by the *Bacillus* probionts, especially if they had not been exposed to the *Bacillus* previously. The probiotic strains used in the present study showed good antibacterial activity against fish, shellfish and human pathogens. *Bacillus* administration has also been shown to increase shrimp survival by enhancing resistance to pathogens by activating both cellular and humoral

immune defences in shrimp (Rengpipat *et al.* 2000). *Bacillus* surface antigens or their metabolites were also reported to act as immunogens for shrimp by stimulating the phagocytic activity of granulocytes (Itami, Kubono, Asano, Tokushige, Takeno, Nishimura, Kondo & Takahashi 1998).

The various mechanisms of probiotic bacterial action might prevent the emergence of resistant strains. The immune systems defend and collectively confer bacterial disease protection. Probiotics are more desirable and environmentally benign compared with the use of antibiotics and chemicals. It is clear from the experiment that the probiotic strains *Bacillus* NL110 introduced through water and feed separately and *Vibrio* NE17 introduced through feed and water significantly enhance the weight gain, SGR, FCR and immunity of *M. rosenbergii* juveniles. It is concluded that the natural bacterial flora associated with the cultured fish and shellfish might have bacterial strains with good probiotic potential, which may be screened and exploited for an environment-friendly and organic mode of aquaculture.

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References

Aly S.M., Abd-El-Rahman A.M., John G. & Mohamed M.F. (2008) Characterization of some bacteria isolated from *Oreochromis niloticus* and their potential use as probiotics. *Aquaculture* **277**, 1–6.

Aly S.M., Mohamed M.F. & John G. (2008) Effect of probiotics on the survival, growth and challenge infection in Tilapia nilotica *(*Oreochromis niloticus*). Aquaculture Research* **39**, 647–656.

Anderson D.P. (1992) Immunostimulant, adjuvants and vaccine carriers in fish: application to aquaculture. *Annual Review of Fish Diseases* **2**, 281–307.

Anderson I.G., Shamsudin M.N. & Nash G. (1989) A preliminary study on the aerobic heterotrophic bacterial flora in giant freshwater prawn, *Macrobrachium rosenbergii*, hatcheries in Malaysia. *Aquaculture* **81**, 213–223.

APHA. (1998) *Standard Methods for the Examination of Water and Wastewater.* American Public Health Association, Washington, DC.

Apun-Molina J.P., Miranda A.S., Gonzalez A.L., Martinez-Diaz S.F. & Rojas-Contreras M. (2009) Effect of potential probiotic bacteria on growth and survival of tilapia *Oreochromis niloticus* L., cultured in the laboratory under high density and sub-optimum temperature. *Aquaculture Research* **40**, 887–894.

Austin B., Stuckey L.F., Robertson P.A.W., Effendi I. & Griffith D.R.W. (1995) A probiotic strain of *Vibrio alginolyticus* effective in reducing diseases caused by *Aeromonas salmonicida*, *Vibrio anguillarum* and *Vibrio ordalii*. *Journal of Fish Diseases* **18**, 93–96.

Balcazar J.L. & Rojas-Luna T. (2007) Inhibitory activity of probiotic *Bacillus subtilis* UTM 126 against *Vibrio* species confers protection against vibriosis in juvenile shrimp (*Litopenaeus vannamei*). *Current Microbiology* **55**, 409–412.

Balcazar J.L., Rojas-Luna T. & Cunningham D.P. (2007) Effect of the addition of four potential probiotic strains on the survival of pacific white shrimp (*Litopenaeus vannamei*) following immersion challenge with *Vibrio parahaemolyticus*. *Journal of Invertebrate Pathology* **96**, 147–150.

Barrow G.I. & Feltham R.K.A. (1993) *Cowan and Steel's Manual for the Identification of Medical Bacteria.* Cambridge University press, New York, USA.

Bell K.L. & Smith V.J. (1993) In vitro superoxide production by hyaline cells of the shore crab *Carcinus maenas* (L.). *Developmental & Comparative Immunology* **17**, 211–219.

Bhan M.K., Bahl R. & Bhatnagar S. (2005) Typhoid and paratyphoid fever. *Lancet* **366**, 749–762.

Cahill M.M. (1990) Bacterial flora of fishes: a review. *Microbial Ecology* **19**, 21–41.

Chaithanya R., Nayak D.K. & Venugopal M.N. (1999) Antibacterial resistance in aquaculture. *INFOFISH International* **6**, 30–32.

Chand R.K. & Sahoo P.K. (2006) Effect of nitrite on the immune response of freshwater prawn *Macrobrachium malcomsonii* and its susceptibility to *Aeromonas hydrophila*. *Aquaculture* **258**, 150–156.

Chen D., Hanna P.J., Altman K., Smith A., Moon P. & Hammond L.S. (1992) Development of monoclonal antibodies that identify *Vibrio* species commonly isolated from infection of human, fish and shellfish. *Applied Environmental Microbiology* **58**, 3694–3700.

Cheng W. & Chen J.C. (1998) Isolation and characterization of *Enterococcus* like bacterium causing muscle necrosis and mortality with *Macrobrachium rosenbergii* in Taiwan. *Diseases of Aquatic Organisms* **34**, 93–101.

Cheng W. & Chen J.C. (2001) Effects of intrinsic and extrinsic factors on the haemocyte profile of the prawn, *Macrobrachium rosenbergii*. *Fish & Shellfish Immunology* **11**, 53–63.

Chugh T.D. (2008) Emerging and re-emerging bacterial diseases in India. *Journal of Bioscience* **33**, 549–555.

Chythanya R., Karunasagar I. & Karunasagar I. (2002) Inhibition of shrimp pathogenic vibrios by a marine *Pseudomonas* I-2 strain. *Aquaculture* **208**, 1–10.

Colorni A. (1985) A study on the bacteria flora of giant prawn, *Macrobrachrium rosenbergii* larva fed with *Artemia salina* nauplii. *Aquaculture* **49**, 1–10.

Dalmin G., Kathiresan K. & Purushothaman A. (2001) Effect of probiotics on bacterial population and health status of shrimp in culture pond ecosystem. *Indian Journal of Experimental Biology* **39**, 939–942.

Decamp O., Moriarty D.J.W. & Lavens P. (2008) Probiotics for shrimp larviculture: review of field data from Asia and Latin America. *Aquaculture Research* **39**, 334–338.

El-Gamal A.A., Alderman D.J., Rodgers C.J., Polglase J.L. & Macintosh D. (1986) A scanning electron microscope study of oxolinic acid treatment of burn spot lesions of *Macrobrachium rosenbergii*. *Aquaculture* **52**, 157–171.

Garriques D. & Arevalo G. (1995) An evaluation of the production and use of a live bacterial isolate to manipulate the microbial flora in the commercial production of *Penaeus vannamei* postlarvae in Ecuador. In: *Swimming Through Troubled Water. Proceedings of Special Session on Shrimp Farming*, San Diego, CA, USA (ed. by C.L. Browdy & J.S. Hopkins), pp. 53–59. World Aquaculture Society, Baton Rouge, LA, USA.

Gatesoupe F.J. (1994) Lactic acid bacteria increase the resistance of turbot larvae, *Scophthalmus maximus*, against pathogenic *Vibrio*. *Aquatic Living Resources* **7**, 277–282.

Gatesoupe F.J. (1997) Siderophore production and probiotic effect of *Vibrio* sp. associated with turbot larvae, *Scophthalmus maximus*. *Aquatic Living Resources* **10**, 239–246.

Gatesoupe F.J. (1999) The use of probiotics in aquaculture. *Aquaculture* **180**, 147–165.

Ghosh S., Sinha A. & Sahu C. (2007) Effect of probiotic on reproductive performance in female live bearing ornamental fish. *Aquaculture Research* **38**, 518–526.

Ghosh S., Sinha A. & Sahu C. (2008) Dietary probiotic supplementation in growth and health of live-bearing ornamental fishes. *Aquaculture Nutrition* **14**, 289–299.

Gibson L.F., Woodworth J. & George A.M. (1998) Probiotic activity of *Aeromonas media* on the Pacific oyster, *Crassostrea gigas*, when challenged with *Vibrio tubiashii*. *Aquaculture* **169**, 111–120.

Gomez-Gil B., Roque A. & Velasco-Blanco G. (2002) Culture of *Vibrio alginolyticus* C7b, a potential probiotic bacterium, with the microalga *Chaetoceros muelleri*. *Aquaculture* **211**, 43–48.

Govoni J.J., Boehlert G.W. & Watanabe Y. (1986) The physiology of digestion in fish larvae. *Environmental Biology of Fishes* **61**, 187–201.

Grisez L. & Ollevier F. (1995) *Vibrio anguillarum* infections in marine fish larviculture. In: *Larvi' 95 - Fish and Shellfish Larviculture Symposium. Gent, Special Publication No. 24* (ed. by P. Lavens, E. Jaspers & I. Roelands), pp. 497. European Aquaculture Society, Oostende, Belgium.

Gullian M., Thompson F. & Rodriguez J. (2004) Selection of probiotic bacteria and study of their immunostimulatory effect in *Penaeus vannamei*. *Aquaculture* **233**, 1–14.

Hamner S., Broadway S.C., Mishra V.B., Tripathi A., Mishra R.K, Pulcini E., Pyle B.H & Ford T.E. (2007) Isolation of potentially pathogenic *Escherichia coli* O157: H7 from the Ganges River. *Applied Environmental Microbiology* **73**, 2369–2372.

Hansen G.H. & Olafsen J.A. (1999) Bacterial interactions in early life stages of marine cold water fish. *Microbial Ecology* **38**, 1–26.

Holmstrom C., Egan S., Franks A., McCloy S. & Kjelleberg S. (2002) Antifouling activities expressed by marine surface associated *Pseudoalteromonas* species. *FEMS Microbiology Ecology* **41**, 47–58.

Holt J.G., Krieg N.R., Sneath P.H.A., Staley J.T. & Willliams S.T. (2000) *Bergy's Manual of Determinative Bacteriology*. Lipponcott Williams & Wilkins, Philadelphia, USA.

Huys L., Dhert P., Robles R., Ollevier F., Sorgeloos P. & Swings J. (2001) Search for beneficial bacterial strains for turbot (*Scophthalmus maximus* L.) larviculture. *Aquaculture* **193**, 25–37.

Irianto A. & Austin B. (2002) Use of probiotics to control furunculosis in rainbow trout, *Oncorhynchus mykiss* (Walbaum). *Journal of Fish Diseases* **25**, 333–342.

Itami T., Kubono K., Asano M., Tokushige K., Takeno N., Nishimura H., Kondo M. & Takahashi Y. (1998) Enhancement of disease resistance of kuruma shrimp, *Penaeus japonicus*, after oral administration of peptidoglycan derived from *Bifidobacterium thermophilum*. *Aquaculture* **164**, 277–288.

Jayaprakash N.S., Pai S.S., Anas A., Preetha R., Philip R. & Singh I.S.B. (2005) A marine bacterium, *Micrococcus* MCCB 104, antagonistic to vibrios in prawn larval rearing systems. *Diseases of Aquatic Organisms* **68**, 39–45.

Keysami M.A., Saad C.R., Sijam K., Daud H.M. & Alimon A.R. (2007) Effect of *Bacillus subtilis* on growth development and survival of larvae *Macrobrachium rosenbergii* (de Man). *Aquaculture Nutrition* **13**, 131–136.

Khuntia C.P., Das B.K., Samantaray B.R., Samal S.K. & Mishra B.K (2008) Characterization and pathogenicity studies of *Vibrio parahaemolyticus* isolated from diseased freshwater prawn, *Macrobrachium rosenbergii* (de Man). *Aquaculture Research* **39**, 301–310.

Klein G., Pack A., Bonaparte C. & Reuter G. (1998) Taxonomy and physiology of probiotic lactic acid bacteria. *International Journal of Food Microbiology* **26**, 103–125.

Kumar R., Mukherjee S.C., Prasad K.P. & Pal A.K. (2006) Evaluation of *Bacillus subtilis* as a probiotic to Indian major carp *Labeo rohita* (Ham.). *Aquaculture Research* **37**, 1215–1221.

Lalitha K.V. & Surendran P.K. (2004) Bacterial microflora associated with farmed freshwater prawn *Macrobrachium rosenbergii* (de Man) and the aquaculture environment. *Aquaculture Research* **35**, 629–635.

Lavilla-Pitogo C.R., Leano E.M. & Paner M.G. (1998) Mortalities of pond-cultured juvenile shrimp, *Penaeus monodon* associated with dominance of luminescent Vibrios in the rearing environment. *Aquaculture* **164**, 337–349.

Le Moullac G., Soyez C., Saulnier D., Ansquer D., Avarre J.C. & Levy P. (1998) Effect of hypoxia stress on the immune response and the resistance to vibriosis of the shrimp

Penaeus stylirostris. *Fish and Shellfish Immunology* **8**, 621–629.

Lightner D.V. (1983) Diseases of cultured Penaeid shrimps. In: *CRC Handbook of Mariculture* (ed. by J.P. McVey), pp. 289–320. CRC Press, Boca Raton, FL, USA.

Lombardi J.V. & Labao V.L. (1991a) Disease and conditioning factors of mortality in larval culture of prawn of the genus *Macrobrachium*. In: *Proceedings of the 3rd Brazilian Symposium on Shrimp Culture*, pp. 401–408. Joao Pessoa, Paraiba, Brazil.

Lombardi J.V. & Labao V.L. (1991b) Diseases and other factors leading to mortality in juveniles and adults belonging to the genus *Macrobrachium*. In: *Proceedings of the 3rd Brazilian Symposium on Shrimp Culture*, pp. 409–419. Joao Pessoa, Paraiba, Brazil.

Martin G.G., Hose J.E., Omori S., Chong C., Hoodbboy T. & McKrell N. (1991) Localization and roles of coagulogen and transglutaminase in hemolymph coagulation in decapod crustaceans. *Comparative Biochemistry & Physiology* **100B**, 517–522.

Martin G., Rubin N. & Swanson E. (2004) *Vibrio parahaemolyticus* and *V. harveyi* cause detachment of epithelium from the midgut trunk of the penaid shrimp *Sicyonia ingentis*. *Diseases of Aquatic Organism* **60**, 21–29.

Ministry of Health & Family Welfare. (2006) *Food safety India: Escherichia coli O157:H7*. Available at http://foodsafetyindia.nic.in/ecolitaq.htm (accessed January 2007).

Moriarty D.J.W. (1996) Microbial biotechnology: a key ingredient for sustainable aquaculture. *INFOFISH International* **4**, 29–33.

Moriarty D.J.W. (1998) Control of luminous *Vibrio* species in penaeid aquaculture ponds. *Aquaculture* **164**, 351–358.

Muroga K., Higashi M. & Keetoku H. (1987) The isolation of intestinal microflora of farmed red seabream (*Pagrus major*) and black seabream (*Acanthopagrus schlegeli*) at larval and juvenile stages. *Aquaculture* **65**, 79–88.

Nejad S.Z., Rezaei M.H., Takami G.A., Lovett D.L., Mirvaghefi A.R. & Shakouri M. (2006) The effect of *Bacillus* spp. bacteria used as probiotics on digestive enzyme activity, survival and growth in the Indian white shrimp *Fenneropenaeus indicus*. *Aquaculture* **252**, 516–524.

New M.B. (1995) Status of freshwater prawn farming: a review. *Aquaculture Research* **26**, 1–54.

Nikoskelainen S., Ouwehand A.C., Bylund G., Salminen S. & Lilius E.M. (2003) Immune enhancement in rainbow trout (*Oncorhynchus mykiss)* by potential probiotic bacteria (*Lactobacillus rhamnosus*). *Fish and Shellfish Immunology* **15**, 443–452.

Panigrahi A., Kiron V., Satoh S., Hirono I., Kobayashi T., Sugita H., Puangkaew J. & Aoki T. (2007) Immune modulation and expression of cytokine genes in rainbow trout *Oncorhynchus mykiss* upon probiotic feeding. *Developmental and Comparative Immunology* **31**, 372–382.

Queiroz J. & Boyd C. (1998) Effects of a bacterial inoculum in channel catfish ponds. *Journal of World Aquaculture Society* **29**, 67–73.

Ramirez R.F. & Dixon B.A. (2003) Enzyme production by obligate intestinal anaerobic bacteria isolated from oscars (*Astronotus ocellatus*), angelfish (*Pterophyllum scalare*) and southern flounder (*Paralichthys lethostigma*). *Aquaculture* **227**, 417–426.

Ravi A.V., Musthafa K.S., Jegathammbal G., Kathiresan K. & Pandian S.K. (2007) Screening and evaluation of probiotics as a biocontrol agent against pathogenic vibrios in marine aquaculture. *Letters in Applied Microbiology* **45**, 219–223.

Ratcliffe N.A., Rowley A.F., Fitzgerald S.W. & Rhodes C.P. (1985) Invertebrate immunity: basic concepts and recent advances. *International Review of Cytology* **97**, 183–350.

Rengpipat S., Phianphak W., Piyatiratitivorakul S. & Menasveta P. (1998) Effects of a probiotic bacterium on black tiger shrimp *Penaeus monodon* survival and growth. *Aquaculture* **167**, 301–313.

Rengpipat S., Rukpratanporn S., Piyatiratitivorakul S. & Menasaveta P. (2000) Immunity enhancement in black tiger shrimp (*Penaeus monodon)* by a probiont bacterium (*Bacillus* S11). *Aquaculture* **191**, 271–288.

Ringo E. & Vadstein O. (1998) Colonization of *Vibrio pelagicus* and *Aeromonas caviae* in early developing turbot (*Scopthalmus maximus* L.) larvae. *Journal of Applied Microbiology* **84**, 227–233.

Riquelme C., Araya R. & Escribano R. (2000) Selective incorporation of bacteria by *Argopecten purpuratus* larvae: implications for the use of probiotics in culturing systems of the Chilean scallop. *Aquaculture* **181**, 25–36.

Rodriguez J., Espinosa Y., Echeverria F., Cardenas G., Roman R. & Stern S. (2007) Exposure to probiotics and β-1,3/1,6-glucans in larviculture modifies the immune response of *Penaeus vannamei* juveniles and both the survival to White Spot Syndrome Virus challenge and pond culture. *Aquaculture* **273**, 405–415.

Shirasaka N., Nishi K. & Shimizu S. (1995) Occurrence of a furan fatty acid in marine bacteria. *Biochimica et Biophysica Acta* **1258**, 225–227.

Singh I.S.B. (1990) Bacterial flora of larvae and larval rearing system of giant fresh water prawn (*M. rosenbergii*). In: *Proceedings of 2nd Kerala Science Congress*, Trivandrum, India (ed. by N. Balakrishnan Nair), pp. 190–194. State Committee on Science, Technology and Environment, Kerala.

Skjermo J. & Vadstein O. (1999) Techniques for microbial control in the intensive rearing of marine larvae. *Aquaculture* **177**, 333–343.

Soderhall K. (1981) Fungal cell wall β-1,3 glucan induce clotting and phenoloxidase attachment ato foreign surface of crayfish haemocyte lysate. *Developmental and Comparative Immunology* **5**, 565–573.

Song Y.L. & Hsieh Y.T. (1994) Immunostimulation of tiger shrimp *Penaeus monodon* haemocytes for generation of microbicidal substances: analysis of reactive oxygen species. *Developmental and Comparative Immunology* **18**, 201–209.

Sugita H., Miyajima C. & Deguchi Y. (1991) The vitamin B_{12}-producing ability of the intestinal microflora of freshwater fish. *Aquaculture* **92**, 267–276.

Sung H.H., Kou G.H. & Song Y.L. (1994) Vibriosis resistance induced by glucan treatment in tiger shrimp *Penaeus monodon*. *Fish Pathology* **29**, 11–17.

Sung H.H., Yang Y.L. & Song Y.L. (1996) Enhancement of microbicidal activity in the tiger shrimp *Penaeus monodon* via immunostimulation. *Journal of Crustacean Biology* **16**, 278–284.

Sung H.H., Hwang S.F. & Tasi F.M. (2000) Responses of giant freshwater prawn (*Macrobrachium rosenbergii*) to challenge by two strains of *Aeromonas* spp. *Journal of Invertebrate Pathology* **76**, 278–284.

Suralikar V. & Sahu N.P. (2001) Effect of feeding probiotic (*Lactobacillus cremoris*) on growth and survival of *Macrobrachium rosenbergii* postlarvae. *Journal of Applied Animal Research* **20**, 117–124.

Tseng D.Y., Ho P.L., Huang S.Y., Cheng S.C., Shiu Y.L., Chiu C.S. & Liu C.H. (2009) Enhancement of immunity and disease resistance in the white shrimp, *Litopenaeus vannamei*, by the probiotic, *Bacillus subtilis* E20. *Fish & Shellfish Immunology* **26**, 339–344.

Uddin M.N. & Al-Harbi A.H. (2005) Quantitative and qualitative bacterial flora of giant freshwater prawn *Macrobrachium rosenbergii* cultured in earthen ponds in Saudi Arabia. *Journal of Aquatic Animal Health* **17**, 244–250.

Venkat H.K., Sahu N.P. & Jain K.K. (2004) Effect of feeding *Lactobacillus* based probiotics on the gut microflora, growth and survival of postlarvae of *Macrobrachium rosenbergii* (de Man). *Aquaculture Research* **35**, 501–507.

Verschuere L., Rombaut G., Sorgeloos P. & Verstraete W. (2000) Probiotic bacteria as biological control agents in aquaculture. *Microbiology and Molecular Biology Reviews* **64**, 655–671.

Vijayan K.K., Bright Singh I.S., Jayaprakash N.S., Alavandi S.V., Somnath Pai S., Preetha R., Rajan J.J.S. & Santiago T.C. (2006) A brackishwater isolate of *Pseudomonas* PS-102, a potential antagonistic bacterium against pathogenic vibrios in penaeid and non-penaeid rearing systems. *Aquaculture* **251**, 192–200.

Wang Y.B. (2007) Effect of probiotics on growth performance and digestive enzyme activity of the shrimp *Penaeus vannamei*. *Aquaculture* **269**, 259–264.

Wang Y.B. & Xu Z.R. (2006) Effect of probiotics for common carp (*Cyprinus carpio*) based on growth performance and digestive enzyme activities. *Animal Feed Science and Technology* **127**, 283–292.

Wang Y.C., Lo C.F., Chang P.S. & Kou G.H. (1998) Experimental infection of white spot baculovirus in some cultured and wild decapods in Taiwan. *Aquaculture* **164**, 221–231.

Woc-Colburn L. & Bobak D.A. (2009) The expanding spectrum of disease due to *Salmonella*: an international perspective. *Current Infectious Disease Reports* **11**, 120–124.

Zhou X., Wang Y. & Li W. (2009) Effect of probiotic on larvae shrimp (*Penaeus vannamei*) based on water quality, survival rate and digestive enzyme activities. *Aquaculture* **287**, 349–353.