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# Growth performance, feed utilization, digestive enzyme activity, innate immunity and protection against *Vibrio harveyi* of freshwater prawn, *Macrobrachium rosenbergii* fed diets supplemented with *Bacillus coagulans*

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**Abstract** The present study was conducted to investigate the effects of dietary supplementation of *Bacillus coagulans* on growth, feed utilization, digestive enzyme activity, innate immune response and disease resistance of freshwater prawn *Macrobrachium rosenbergii*. Three treatment groups (designated as *T*1, *T*2 and *T*3) and a control group (*C*), each in triplicates, were established. The prawn in the control were fed a basal diet and those in *T*1, *T*2 and *T*3 were fed basal diet containing *B. coagulans* at $10^5$, $10^7$ and $10^9$ cfu $g^{-1}$, respectively. After 60 days, growth performance and feed utilization were found significantly higher ($P < 0.05$) in prawn fed *T*3 diet. The specific activities of protease, amylase and lipase digestive enzymes were significantly higher ($P < 0.05$) for *T*3. Innate immunity in terms of lysozyme and respiratory burst activities were significantly elevated ($P < 0.05$) in all the probiotic treatment groups as compared to control. Challenge study with *Vibrio harveyi* revealed significant increase ($P < 0.05$) in disease resistance of freshwater prawn in *T*2 and *T*3 groups. The results collectively suggested that supplementation of *B. coagulans* as probiotic in the diet at approximately $10^9$ cfu $g^{-1}$ can improve the growth performance, feed utilization, digestive enzyme activity, innate immune response and disease resistance of freshwater prawn.

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**Keywords** Freshwater prawn · *Bacillus coagulans* · Probiotic · Growth performance · Innate immunity · Enzyme activity · Disease resistance

## Introduction

*Macrobrachium rosenbergii,* the giant freshwater prawn is considered as a candidate species among the crustaceans for aquaculture in different parts of tropical and subtropical regions. It has cultural importance due to its fast growth rate, high demand in national and international markets and tolerance to aquatic environmental condition (Ranjeet and Kurup 2002; New 2005; Gupta et al. 2007).

In large-scale production facilities, aquatic animals are exposed to stressful conditions. The increased intensity of aquaculture has led to a high number of disease outbreak with an increasing range of pathogens as a result in serious economic losses (Verma and Gupta 2015). The abuse of disinfectants, pesticides, and antimicrobial drugs has caused the evolution of resistant strains of bacteria and concern of the society (Esiobu et al. 2002). Probiotic is the use of microbial supplements to benefit their host (Fuller 1989). Thus, the use of microorganisms as probiotics in the culture of aquatic organisms is increasing with the demand for more environment-friendly aquaculture practice (Gatesoupe 1999).

The most commonly used probiotics in aquaculture belong to gram-positive spore-forming *Bacillus* spp. (Wang et al. 2008; Gupta and Dhawan 2011, 2013; Gupta et al. 2014). *Bacillus* preparations are resistant to the environment, have a long lasting shelf life and the beneficial roles of these bacterial species in the aquaculture field are well established (Gatesoupe 1999; Wang et al. 2008). Within the *Bacillus* genus, *Bacillus coagulans* has potent biocontrol ability against a number of fish pathogens which are administered by enrichment of live feeds, added to the diets or to culture water (Panigrahi et al. 2005; Kumar et al. 2006; Kim and Austin 2006; Gupta et al. 2014). The effects of probiotic *B. coagulans* have been linked to modulation of gut microbiota and establishment of the beneficial microorganisms, higher specific and total digestive enzyme activities in the brush-border membrane which increases the nutrient digestibility and feed utilization (Balcazar et al. 2006; Kesarcodi-Watson et al. 2008). The digestive enzymes in fish have been studied by several workers (Kawai and Ikeda 1972; Das and Tripathi 1991). Furthermore, the immunostimulatory effects of these probiotic bacteria against *Aeromonas hydrophila* infection in fish have been demonstrated (Aly et al. 2008; Kumar et al. 2008; Zhou et al. 2010; He et al. 2011). However, there is lack of studies regarding the use of spore-forming lactic acid-producing bacteria such as *B. coagulans* as a probiotic in freshwater prawn.

Keeping this in view, the principal objective of the current study was to evaluate the effects of dietary administration of *B. coagulans* on the changes in digestive enzyme activities and innate immune responses of *M. rosenbergii*. In addition, effect on water quality, growth performance and survival was also investigated. To the best of our information, the present study is the first to evaluate the potential of probiotic *B. coagulans* in relation to growth performance, feed utilization, digestive enzyme activity and immune response in commercially important crustacean *M. rosenbergii.*

## Materials and methods

### Probiotic bacteria

*Bacillus coagulans strain* (MTCC 9872) was procured from the Indian Institute of Microbial Technology, Chandigarh, India, and used as potential probiotic. The strain was revived in nutrient broth for 48 h at 37 °C. To confirm the purity of strain, colonies were identified on the basis of their morphological, Gram's staining and biochemical characteristic using bacterial identification kit (HiMedia, India). Cell density was calculated using spectrophotometer (Hach DR2800, USA) at 600 nm and values were correlated with colony-forming unit (CFU) counts using serial dilution and spread plating on their respective agar. The quantified bacteria were centrifuged, and suspensions were made with phosphate-buffered saline (PBS, pH 7.5). The suspended form was used for feed preparation as required.

### Antagonism study and evaluation of possible harmful effect of probiotic strain on prawn

Agar well diffusion test was conducted to study antagonistic activity of *B. coagulans* following a previously described method (Balcazar et al. 2007) with some modification (Gupta et al. 2014). The antagonistic activity was examined against indicator pathogenic bacteria like *A. hydrophila* (MTCC 1739) and *Vibrio harveyi* (MTCC 7771). For the determination of possible harmful effect of *B. coagulans*, the culture grown in broth was centrifuged at 1000*g* for 10 min at 4 °C, washing twice and re-suspending in PBS. Volume (20 µl) of each suspension containing approximately $10^5$ cells $ml^{-1}$, as determined by a haemocytometer slide at a magnification of 400 on a Leica semi-motorized research microscope (DM3000; Germany), was injected into the third abdominal segment into group of 20 prawns. Another group of twenty prawns were injected with normal saline

**Table 1** Composition and proximate analysis of basal diet

| Ingredients | Composition (%) | Proximate analysis | Composition (% dry matter) |
|---|---|---|---|
| Soybean meal | 40 | Dry matter | 91.12 |
| Fish meal | 30 | Crude protein | 32.67 |
| Mustard oil cake | 10 | Lipid | 6.15 |
| Rice bran | 10 | Ash | 11.23 |
| Corn starch | 5 | Organic matter[b] | 79.95 |
| Sunflower oil | 4 | Total carbohydrate[c] | 41.13 |
| Vitamin and mineral mixture[a] | 1 | Gross energy (kcal 100 $g^{-1}$) | 387.5 |

[a] Vit and min. mixture: every gram of vitamin–mineral mixture provided vitamin A, 8000 IU; vitamin $B_2$, 2.8 mg; vitamin $B_{12}$, 5 µg; vitamin $D_3$, 1500 IU; vitamin E, 5 mg; vitamin $K_3$, 5 mg; vitamin PP, 12.5 mg; D calcium pantothenate, 5 mg; copper sulphate, 0.7 mg; zinc sulphate, 2.5 mg; ferrous sulphate, 6.2 mg; potassium iodide, 0.4 mg; manganese sulphate, 3.8 mg and sorbitol, 20 mg

[b] Organic matter = dry matter − total ash

[c] Total carbohydrates = organic matter − (crude protein + total lipid)

solution (NSS), as controls, to observe the symptoms of diseases. After 10 days, survivors were examined for evidence of disease.

## Experimental diets

The formulation and proximate analysis of the basal diet is presented in Table 1 and was prepared according to Gupta and Dhawan (2012) and Gupta et al. (2014). The basal diet was used as control (*C*). In the three experimental diets *T*1, *T*2, and *T*3, probiotic *B. coagulans* suspension was added at a final dose of $10^5$, $10^7$ and $10^9$ cfu $g^{-1}$, respectively. To achieve accurate final concentrations in the diet, the bacterial suspension was slowly added to dough, with gradual mixing in the laminar airflow chamber. Each diet was then passed through a mincer, and the resulting pellets were dried in an oven using an air blower at 37 °C until the moisture levels were around 10 %. After drying, the pellets were broken up and sieved into pellets of approximate size and stored at −20 °C in sealed plastic ziploc bags until used.

The survival of the supplemented bacteria in the diet was assessed following storage at 4 °C on weekly basis for two weeks. One gram of the diet was homogenized in 9.0 ml of sterile saline solution, and serial dilutions to $10^{-5}$ were prepared and 0.2 ml was spread onto duplicate plates of nutrient agar. The colonies were counted after incubation for 24–48 h at 30 °C. Based on the data of survival of probiotic, feeds were prepared on weekly basis to ensure high probiotic levels in the supplemented feed.

## Experimental animal and conditions

Live specimens of healthy freshwater prawn were provided by the College of Fisheries, GADVASU, Ludhiana (Punjab), India, and the experiment was conducted at the same place. Twelve experimental fibreglass tanks of 500-l capacity were prepared before stocking of prawn. A 5-in.-thick layer of soil was spread on the bottom of each tank to facilitate breakdown of organic matter. Over the soil, lime was applied at the rate of 150 kg $ha^{-1}$ seven days before the tanks were filled with water to a depth of 20 cm. The tanks were also provided with PVC pipes of 10 × 20 cm size as a substrate to increase the surface area for prawn. Two hundred and forty juvenile prawns were weighted, randomly distributed in tanks containing freshwater and acclimatized for seven days prior to the experiment.

One group served as the control (*C*) and was fed basal diet during the entire experimental period. The other three groups were fed *B. coagulans*-supplemented diets at three different doses $10^5$ (*T*1), $10^7$ (*T*2) and $10^9$ (*T*3) cfu $g^{-1}$ feed, until the end of the experiment. Experiment was conducted in triplicate for 60 days. Prawns were fed their specific diets twice a day at 5 % of the body weight. The mortality rate of prawn in each tank was recorded daily.

## Water quality analysis

Temperature, pH, dissolved oxygen (DO), free carbon dioxide ($FCO_2$), ammonium ($NH_4$-N) and nitrite ($NO_2$-N) as water quality parameters were measured fortnightly before water exchange by following the standard procedures (APHA 1989).

## Biometry data

At the end of 60 day experiment, the final body weight, weight gain, specific growth rate, survival, feed conversion ratio (FCR) and protein efficiency ratio (PER) were calculated by using following formulae as described by Gupta et al. (2007):

$$\text{Specific growth rate (SGR)} = 100(\log_e \text{final body weight} - \log_e \text{initial body weight}) / \text{culture period (days)}$$

$$\text{Feed conversion ratio (FCR)} = \text{total dry feed fed (g)} / \text{total live weight gain (g)}$$

$$\text{Protein efficiency ratio (PER)} = \text{weight gain/protein intake}$$

## Digestive enzyme assay

Five prawns from each tank were randomly collected for digestive enzyme assay after the termination of experimental trial. The gastrointestinal tract (GIT) of each prawn was removed aseptically and then immediately packed and stored in −20 °C until analysis. Enzymatic extracts were prepared according to Ding et al. (2004). Briefly, the prawns were rinsed with distilled water and deshelled. A brief cut was given to obtain intact GIT at 4 °C and rinsed with cold distilled water. The total GIT content was homogenized in PBS (phosphate-buffered saline, pH 7.5; 1:10) in a handheld homogenizer at 4 °C, and homogenates were centrifuged at 5000×*g* for 20 min at 4 °C. The supernatant was retained and stored at −20 °C to analyze different enzymes.

The total protein content was measured using in diluted homogenates following the Lowry method (Lowry et al. 1951) using bovine serum albumin as a standard. Protease activity was evaluated according to Anson (1938) using Folin phenol reagent, and amylase activity was measured according to Jiang (1982) and Worthington (1993) using iodine solution to reveal non-hydrolysed starch. Lipase activity was determined based on the measurement of fatty acid released due to enzymatic hydrolysis of triglycerides in stabilized emulsion of olive oil (Borlongan 1990; Jin 1995). Enzyme activities were measured as the change in absorbance using spectrophotometer and expressed as specific activity (U $mg^{-1}$ protein).

## Immune assay

For innate immune response, six prawns were randomly removed from each tank at the end of experiment. Haemolymph (50–100 µl) was withdrawn from the ventral sinus at the base of the first abdominal segment of each prawn into a 26-G needle and a 1-ml syringe containing an anticoagulant solution (13 mM tri-sodium citrate, 0.34 M sodium chloride and 10 mM EDTA-$Na_2$, at a pH of 7.55 with osmolality adjusted to 780 mosM $kg^{-1}$ using 0.115 M glucose) (Liu and Chen 2004). The anticoagulant–haemolymph of prawns was pooled and gently mixed in sterile eppendorf tube immediately. All assays of immune parameters were conducted in triplicates.

### *Lysozyme activity*

The lysozyme activity level was measured using the turbidimetric assay following Sankaran and Gurnani (1972). Lysozyme activity was expressed as units/ml, where one unit

was defined as the reduction in absorbance of 0.001/min. Lyophilized hen egg white lysozyme (HiMedia, India) was used to develop a standard curve.

*Respiratory burst*

The respiratory burst or super oxide generation ($O_2^-$) assay of haemolymph was carried out following the protocol of Anderson and Siwicki (1995) using nitroblue tetrazolium (HiMedia, India) as standard. The optical density (OD) of the supernatant was measured at 540 nm in the microplate reader (Model: Infinite M200 PRO, Tecan, Switzerland). *N*,*N*-dimethyl formamide (Qualigens, Fisher Scientifics, India) was used as blank. The results were expressed as an increase in absorbance 100 µl $HL^{-1}$.

### Experimental infection/bacterial challenge

After 60 days of the feeding period, an experimental infection was induced in prawn with the pathogenic bacterium, *V. harveyi* (MTCC 7771). *V. harveyi* was grown overnight in nutrient broth, and the concentration was adjusted to $10^7$ cfu $ml^{-1}$ using NSS. A total of sixty prawn (five from each replicate) in the intermoult stage were collected from the treatment and control groups and injected with 20 µl of the bacterial suspension into the ventral sinus of the third abdominal segment resulting $10^6$ cfu $prawn^{-1}$. Immediately after injection, prawns were transferred into the 50-l glass aquaria. The experiment was conducted in duplicate. A group of untreated prawn with *B. coagulans*-supplemented diet, which was injected with NSS (20 µl), served as positive/unchallenged control (PC). During the experimental infection, prawns were fed their specific diets as previously described. The mortality was monitored daily for up to 7 days.

### Statistical analysis

Data obtained from the present experiment were analysed using one-way analysis of variance (ANOVA) to determine if significant differences ($P < 0.05$) existed among treatment means. The percentage date were transformed to square-root arcsine values before the statistical analysis. Tukey's test was used to compare means between individual treatments. Statistical analyses were performed using SPSS for Windows version 16.0.

## Results

### Safety of probiotic strain

The results of the in vitro antagonistic study showed that the *B. coagulans* induced an inhibition zone against *A. hydrophila* and *V. harveyi* of 4.1 and 6.5 mm, respectively. The results indicated that the probiotic strain have potential antagonistic activity against pathogenic bacteria. There were no signs of disease in probiotic-injected prawn as neither mortalities nor morbidities were observed during 10 days of probiotic-challenge examination. This confirms the non-pathogenicity of *B. coagulans* in freshwater prawn.

## Water quality

The effect of probiotic, *B. coagulans,* on water quality parameters in freshwater prawn tanks are presented in Table 2. The average temperature, pH and DO in treated tanks ranged from 27.9 ± 2.8 to 28.6 ± 3.1 °C, 7.7 ± 2.9 to 7.8 ± 2.5 and 7.4 ± 2.1 to 7.6 ± 3.9 mg $l^{-1}$, respectively, while that of control were 28.6 ± 2.5 °C, 7.8 ± 2.8 and 7.3 ± 2.4 mg $l^{-1}$, respectively. There was no significant difference among control and probiotic-treated tanks ($P > 0.05$) during the experimental period in terms of temperature, pH and DO. Free carbon dioxide ($FCO_2$) remained nil throughout the experimental period in all treatments including control. Moreover, no significant difference were observed ($P > 0.05$) throughout the experimental period in $NH_4$-N concentration although the higher level was determined in control compared with *T*1, *T*2 and *T*3. As for the $NO_2$-N concentration, analysis showed no difference ($P > 0.05$) in group treated with *B. coagulans*, as compared with the control although $NO_2$-N concentration in *T*3 had a relatively lower content (Table 2).

## Survival, growth and feed utilization

The growth performance and associated nutritional indices are presented in Table 3. Prawn fed with *B. coagulans* showed significantly higher ($P < 0.05$) survival rate over the control (80.0 ± 11.5 %) after 60 days of culture. After 60 days of feeding, the growth performance and feed utilization of the prawn groups fed probiotic incorporated diet was significantly improved compared to those of prawn fed control diet. Inclusion of probiotic in diet significantly ($P < 0.05$) enhanced final body weight, specific growth rate, protein efficiency ratio as compared to control. Prawn fed C (control) diet showed lowest significant final body weight, specific growth rate and protein efficiency ratio (Table 3). Improved non-significant differences were found in feed conversion ratio of prawn fed probiotic-supplemented diets; however, inferior significant value was recorded in control group.

**Table 2** Changes in the physico-chemical properties of water during 60-day experimental trial on supplementation of *B. coagulans* in the diet of *M. rosenbergii*

| Parameter | Treatment | | | |
|---|---|---|---|---|
| | Control (*C*) | *T*1 | *T*2 | *T*3 |
| Temperature (°C) | 28.6 ± 2.5[a] | 27.9 ± 2.8[a] | 28.5 ± 2.6[a] | 28.6 ± 3.1[a] |
| pH | 7.8 ± 2.8[a] | 7.6 ± 2.3[a] | 7.8 ± 2.5[a] | 7.7 ± 2.9[a] |
| DO (mg $l^{-1}$) | 7.3 ± 2.4[a] | 7.6 ± 3.9[a] | 7.4 ± 2.1[a] | 7.5 ± 2.8[a] |
| $FCO_2$ (mg $l^{-1}$) | Nil | Nil | Nil | Nil |
| $NH_4$-N (mg $l^{-1}$) | 0.031 ± 0.005[a] | 0.028 ± 0.003[a] | 0.027 ± 0.004[a] | 0.028 ± 0.005[a] |
| $NO_2$-N (mg $l^{-1}$) | 0.010 ± 0.004[a] | 0.009 ± 0.002[a] | 0.009 ± 0.001[a] | 0.008 ± 0.001[a] |

Values are mean of triplicate groups and presented as mean ± SE. Values with different superscripts in the same row are significantly different ($P < 0.05$). Treatment *T*1, *T*2, and *T*3 containing probiotic *B. coagulans* at a final dose of $10^5$, $10^7$ and $10^9$ cfu $g^{-1}$ feed, respectively

**Table 3** Growth performance and feed utilization of *M. rosenbergii* fed 32 % crude protein diet containing different levels of *B. coagulans* as probiotic for 60 days

| Treatment | Control (*C*) | *T*1 | *T*2 | *T*3 |
|---|---|---|---|---|
| Initial body weight (g) | $2.4 \pm 0.35^{a}$ | $2.4 \pm 0.35^{a}$ | $2.4 \pm 0.35^{a}$ | $2.4 \pm 0.35^{a}$ |
| Final body weight (g) | $8.80 \pm 0.26^{b}$ | $11.83 \pm 1.91^{a}$ | $11.88 \pm 0.08^{a}$ | $13.21 \pm 0.21^{a}$ |
| Specific growth rate (% $d^{-1}$) | $2.17 \pm 0.3^{b}$ | $2.66 \pm 0.2^{a}$ | $2.67 \pm 0.2^{a}$ | $2.84 \pm 0.3^{a}$ |
| Survival (%) | $80.0 \pm 11.5^{b}$ | $100 \pm 0.0^{a}$ | $100 \pm 0.0^{a}$ | $100 \pm 0.0^{a}$ |
| Feed conversion ratio | $1.68 \pm 0.11^{a}$ | $1.46 \pm 0.13^{b}$ | $1.48 \pm 0.09^{b}$ | $1.42 \pm 0.10^{b}$ |
| Protein efficiency ratio | $1.54 \pm 0.17^{b}$ | $1.95 \pm 0.21^{a}$ | $1.93 \pm 0.14^{a}$ | $1.99 \pm 0.23^{a}$ |

Values are mean of triplicate groups and presented as mean ± SE. Values with different superscripts in the same row are significantly different ($P < 0.05$). Treatment *T*1, *T*2, and *T*3 containing probiotic *B. coagulans* at a final dose of $10^5$, $10^7$ and $10^9$ cfu $g^{-1}$ feed, respectively

## Digestive enzyme activity

Specific protease activity in prawn was significantly different between probiotic treatments and control (Table 4). The highest protease activity was observed in *T*3 followed by *T*1 and *T*2. There was no significant difference ($P < 0.05$) between *T*2 and control treatments in protease activity. Amylase activity in *T*3 was significantly higher ($P < 0.05$) than *T*2 and *T*1. As for the lipase activity of prawn, assay showed significant difference ($P < 0.05$) in treatments with different levels of *B. coagulans* over the control. However, a concentration-dependant response of probiotic strains in *T*3 and *T*1 was not observed in the present study. Also, there was no significant difference ($P < 0.05$) in lipase activity between the *T*1 and *T*2.

## Immune response

The innate immune response parameters are presented in Table 5. There was no significant difference among probiotic fed dietary treatments in lysozyme activity ($P > 0.05$). Prawn group fed diet supplemented with *B. coagulans* exhibited the highest lysozyme activity than control. The respiratory burst activities (RBA) in the haemolymph showed significant differences among the experimental groups. Prawn fed probiotic-supplemented diets showed significant higher RBA than control (*C*) fed basal diet only. However, the differences among the probiotic fed prawn were insignificant ($P > 0.05$).

**Table 4** Intestinal changes in the digestive enzyme activity of *M. rosenbergii* fed diet containing different levels of *B. coagulans* as probiotic for 60 days

| Enzyme activity (U $mg^{-1}$ protein) | Control (*C*) | *T*1 | *T*2 | *T*3 |
|---|---|---|---|---|
| Protease | $0.18 \pm 0.03^{c}$ | $0.25 \pm 0.02^{b}$ | $0.22 \pm 0.01^{bc}$ | $0.35 \pm 0.04^{a}$ |
| Amylase | $0.143 \pm 0.05^{c}$ | $0.167 \pm 0.02^{b}$ | $0.168 \pm 0.04^{b}$ | $0.340 \pm 0.06^{a}$ |
| Lipase | $0.42 \pm 0.07^{c}$ | $0.62 \pm 0.08^{ab}$ | $0.58 \pm 0.06^{b}$ | $0.65 \pm 0.07^{a}$ |

Values are mean of triplicate groups and presented as mean ± SE. Values with different superscripts in the same row are significantly different ($P < 0.05$). Treatment *T*1, *T*2, and *T*3 containing probiotic *B. coagulans* at a final dose of $10^5$, $10^7$ and $10^9$ cfu $g^{-1}$ feed, respectively

**Table 5** Lysozyme and respiratory burst activity of freshwater prawn fed diet containing different levels of *B. coagulans* as probiotic for 60 days

| Innate immune response | Control (*C*) | *T*1 | *T*2 | *T*3 |
|---|---|---|---|---|
| Lysozyme (U $ml^{-1}$ $min^{-1}$) | $26.16 \pm 4.46^{b}$ | $54.63 \pm 4.6^{a}$ | $58.21 \pm 3.61^{a}$ | $50.1 \pm 2.7^{a}$ |
| RBA (OD 540 nm) | $0.451 \pm 0.10^{b}$ | $0.813 \pm 0.09^{a}$ | $0.805 \pm 0.058^{a}$ | $0.761 \pm 0.022^{a}$ |

Values are mean of triplicate groups and presented as mean ± SE. Values with different superscripts in the same row are significantly different ($P < 0.05$). Treatment *T*1, *T*2, and *T*3 containing probiotic *B. coagulans* at a final dose of $10^5$, $10^7$ and $10^9$ cfu $g^{-1}$ feed, respectively

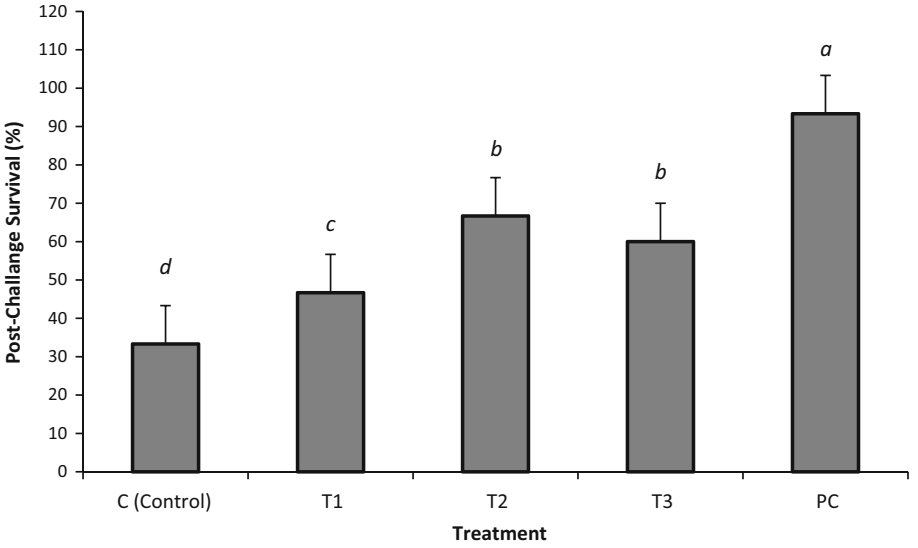


**Fig. 1** Survival rate of freshwater prawn *M. rosenbergii* fed diet containing different levels of *B. coagulans* as probiotic after challenge with *V. harveyi*. Control (*C*): prawn fed with basal diet. *T*1, *T*2 and *T*3: prawn fed with basal diet supplemented with different levels of *B. coagulans* @ $10^5$, $10^7$ and $10^9$ cfu $g^{-1}$ feed, respectively. PC: positive/unchallenged control. Data are presented as mean ± SE. *Different superscripts* indicate statistically significant differences ($P < 0.05$) between treatments

## Resistance against infection

The challenge test revealed that long-term dietary administration of *B. coagulans*-supplemented diet enhanced the resistance of *M. rosenbergii* to infection (Fig. 1). Prawn mortality after injection of *V. harveyi* increased significantly ($P < 0.05$) with time and observed after 24 h of post injection and onwards. Significant higher post-challenge survival ($P < 0.05$) was observed in the prawn fed diet containing *B. coagulans*. The prawn fed the control diet exhibited the lowest survival (33.33 %) followed by *T*1 (46.67 %); however, highest was observed in positive/unchallenged control (93.33 %) (Fig. 1).

## Discussion

*Bacillus* species are gaining more and more importance and are widely used in aquaculture due to their longer stability, easy preparedness, antagonistic effects on pathogens and enhancement of immunity (Hong et al. 2005; Gupta et al. 2014). The results of the in vitro antibacterial activity of the probiotic strain used against the pathogenic *A. hydrophila* and *V. harveyi* revealed that *B. coagulans* has probiotic potential. Previous works have established that *Bacillus* like *B. coagulans* and *B. licheniformis* and *Paenibacillus* like *P. polymyxa and P. thiaminolyticus* produce antibacterial compounds, such as polymyxin, octopytin and baciphelacin (Slepecky and Hemphill 1992).

It is necessary to provide freshwater prawn with a healthy environment, and probiotics has a great deal of potential (Zhou et al. 2009). Zhou et al. (2009) found that the treatment of *P. monodon* with different levels of *B. coagulans* did not significantly affected water quality compared with the control. However, Wang et al. (2005) investigated the effect of commercial probiotic on water quality in *P. vannamei* ponds, and the results showed that probiotics could significantly reduce the concentrations of nitrogen and phosphorous in pond water. In this study, the use of *B. coagulans* in freshwater prawn as feed additive had shown inconsistent results. In contrast, there was no obvious effect of probiotic on the water quality during the study. This result may be explained by the good water quality and culture conditions in this study in contrast to theirs. In fact, the pH and DO values and concentrations of ammonium and nitrite determined in this study were stable and within acceptable ranges, as required for the culture of freshwater prawn (Gupta et al. 2007; Gupta and Dhawan 2013).

The supplementation of *B. coagulans* in the diet resulted in improved growth performance of freshwater prawn. These results are supported by feeding of *Bacillus* spp. in the Indian white shrimp (*Fenneropenaeus indicus)* (Ziaei-Nejad et al. 2006); pacific white shrimp (*Litopenaeus vannamei*) (Wang 2007; Far et al. 2009; Wang and Gu 2010); *M. rosenbergii* (Keysami et al. 2007; Gupta and Dhawan 2012, 2013); and *Penaeus monodon* (Rahiman et al. 2010). Kumar et al. (2012) observed no significant differences in the survival, whereas significant increase in growth parameters and decrease in FCR were observed in *M. rosenbergii* fed diets supplemented with different concentrations of *B. licheniformis*. Rahiman et al. (2010) reported an increase in the weight gain and SGR in *M. rosenbergii* fed diet supplemented with *Bacillus* species without any significant increase in the survival. In contradiction with our results, Shariff et al. (2001) found that the treatment of *P. monodon* with a commercial *Bacillus* probiotic did not significantly increase survival. However, it is difficult to directly assess different studies using probiotics, because the efficacy of a probiotic application depended on many factors (Lara-Flores 2011; Wang et al. 2012) such as species composition and viability, administration level, mode and frequency of application, diet composition, prawn age and environmental stress factors.

Prawn fed diet supplemented with *B. coagulans* showed remarkable increase in digestive enzyme activities. Lovett and Felder (1990) and Kamarudin et al. (1994) investigated that the shrimp digestive system was activated during larval and post-larval stages, where the probiotics would have the utmost effect. Moreover, a wide variety of exoenzymes are secreted by bacteria, predominantly by members of the genus *Bacillus* (Moriarty 1996, 1998). Furthermore, the exogeneous enzymes secreted by the probiotic bacteria would contribute a little to the total enzyme activity of the gut (Ding et al. 2004; Ziaei-Nejad et al. 2006). Hence, we could not distinguish between activity due to exoenzymes synthesized by probiotic bacteria and activity due to endoenzymes

synthesized by prawn. In the present study, different concentrations of probiotic had different consequences on enzyme activity. The protease, amylase and lipase activity increased significantly with increase in concentration of probiotic. The higher level of enzyme activity obtained with diets containing *B. coagulans* might have improved the digestion of protein, starch, fat and cellulose, which in turn led to enhanced digestion and proper absorption of food that serves as the reason for better growth observed with probiotic-supplemented diets.

Lysozyme in serum prevents adherence and colonization of microorganisms, thus helps in circumvent the disease (Alexander and Ingram 1992). In the present study, tremendous increase of lysozyme activity were demonstrated in all treatments fed *Bacillus* spp. compared to control. Similar results were obtained by Gullian et al. (2004) using *Bacillus*, as a probiotic, that showed better immunostimulatory features in *L. vannamei*. Our study showed that prawn groups fed with different levels of probiotic had significantly ($P < 0.05$) higher respiratory bursts than the control group, confirming that innate immune response was enhanced in prawn fed diets containing *B. coagulans*. We assume that *B. coagulans* inhabits for long term in probiotic-treated prawn guts and hence provides a long-term immune stimulation for freshwater prawn. Similar trend in stimulation of respiratory burst activity after dietary probiotic supplementation has been reported in *M. rosenbergii* fed *Bacillus* spp. (Rahiman et al. 2010) and *L. vannamei* fed *Arthrobacter species* (Li et al. 2008).

Probiotics help in achieving natural resistance and high survivability of fish (Abraham et al. 2007). The dietary supplementation of *B. coagulans* in the present study showed good antibacterial activity against *V. harveyi* and significantly increased survival rate of freshwater prawn after challenged with pathogenic bacteria. In a previous study, *Bacillus* administration has also been shown to increase survival by enhancing resistance to pathogens by acting both cellular and humoral immune defence in shrimp and prawn (Rengpipat et al. 2000; Gupta and Dhawan 2013). *Bacillus* surface antigens or their metabolites were also reported to act as immunogens for shrimp by stimulating the phagocytic activity of granulocytes (Itami et al. 1998). Protection was achieved in eel (*Anguilla anguilla*) and Indian carp (*Catla catla*) against *A. hydrophila* infection after the fish were fed with diet supplemented with *B. amyloliquefaciens* (Cao et al. 2011; Das et al. 2013). Such enhancement in prawn disease resistance can be partially due to the facilitated innate immune responses of fed prawn where significantly higher lysozyme and RBA ($P < 0.05$) level were obtained. The results indicate the involvement of an activated and prolonged immune status of freshwater prawn to resist *V. harveyi* infection.

## Conclusion

The results of present study demonstrated that the use of *B. coagulans* at approximately $10^9$ cfu $g^{-1}$ as a dietary probiotic could significantly improve survival rate, growth performance, feed utilization and digestive enzyme activity of freshwater prawn *M. rosenbergii.* The negative environment effects on freshwater prawn could thus be mitigated through the use of *B. coagulans*, which is known to enhance immune activities and disease resistance against *V. harveyi* as seen in the present investigation.

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