Evaluation of different probiotic strains for growth performance and immunomodulation in Pacific white shrimp *Penaeus vannamei* Boone, 1931

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ABSTRACT

The use of antibiotics in aquaculture can prevent certain diseases, however, its use is highly restricted due to several environmental and human health problems like development of antibacterial resistance. Probiotics are widely used for improving production of aquatic animals by means of improving water quality as well as by nutritional and immune modulation in animals thus, helping in preventing diseases. The present study was aimed to evaluate different strains of probiotics *viz.,* a commercial probiotic, *Bacillus subtilis, Enterococcus* sp*., Lactobacillus casei, Lactobacillus rhamnosus, Saccharomyces cerevisiae* and *Saccharomyces boulardii* on the growth, microbial load and immunomodulatory performance of the Pacific white shrimp, *Penaeus vannamei*. Seven probiotic feeds each containing 5×10^9 CFU ml^{-1} of respective probiotics kg^{-1} of feed were prepared by top coating on a pellet feed containing 35% protein and duration of the experiment was 75 days. All of the 6 selected strains of probiotics except the commercial one influenced the growth significantly when fed to the shrimp, compared to control group. Specific growth rate (SGR), average daily growth (ADG) and survival rate were observed to be higher in probiotic fed groups. Maximum growth was recorded in *S. cerevisiae* fed groups (8.05 ± 0.21 g), followed by *B. subtilis* (7.65 ± 0.21 g), while the control animals showed an average growth of 4.85 ± 0.49 g with percentage improvement in the range of 50-95% when compared to all other treatments. Total heterotrophic count significantly ($p<0.05$) increased in the rearing water of *B. subtilis* treated group ($8.995\pm 0.021 \times 10^3$ cfu ml^{-1}) when compared to that of control ($5.475\pm 0.003 \times 10^3$ cfu ml^{-1}) and total vibrio load was greatly reduced in *B. subtilis* ($1.42 \pm 0.04 \times 10^3$ cfu ml^{-1}) and *S. cerevisiae* ($1.47 \pm 0.01 \times 10^3$ cfu ml^{-1}) treated water compared to control ($4.265 \pm 0.06 \times 10^3$ cfu ml^{-1}). Non-specific immunity in terms of total haemocyte count (THC) was found to be significantly ($p<0.05$) higher in *B. subtilis* ($12.4\pm0.8 \ 10^6$ cells ml^{-1}) treated group while higher ($p<0.05$) prophenoloxidase (pro PO) activity was recorded in *S. cerevisiae* (0.132 ± 0.001 units min^{-1} mg protein^{-1}) and *B. subtilis* (0.130 ± 0.002 units min^{-1} mg protein^{-1}) treated groups. The probiotic effect was found to be beneficial for better growth and immunomodulation, which was however found to be strain-specific.

Keywords: Immunity, *Penaeus vannamei,* Probiotics, Survival rate

Introduction

Shrimp farming has been plagued with infectious disease outbreaks from many bacterial and viral pathogens. Control strategies have been developed for emerging diseases for improving animal health, better production and environmental friendly sustainable culture. Antibiotics have been used in aquaculture for control of infectious diseases but these have enormous environmental, economic and social concerns. Probiotics are beneficial bacteria that promote the wellbeing of a host animal and contribute to the direct and/or indirect protection of host animals against harmful bacteria. These beneficial microbes favour the host by competitive inhibition of pathogenic microbes. The application of probiotic bacteria in aquaculture has tremendous scope, outputs and glorious future. Probiotic organisms are found to be the richest source of many beneficial products, such as

vitamins, minerals and trace elements and important digestive enzymes thereby having nutritional benefits (Wang *et al.*, 2005; Vo Minh Son *et al.*, 2009). Probiotic treatment results in better survival, growth, disease resistance and improve the protective response especially during the larval stages in shrimps and fishes (Gullian *et al.*, 2004).

Studies in fish and shellfishes have shown the beneficial effects of probiotics in enhancing the growth rate and survival (Panigrahi *et al.*, 2005, 2007; Karunasagar, 2007). There are various studies elucidating the beneficial use of probiotics (bacterins) in crustaceans with encouraging results (Itami *et al.*, 1989, 1991; Karunasagar *et al.*, 1994; Devaraja *et al.*, 2002; Azad *et al.*, 2005). Several studies demonstrated improved survival and growth rate of shrimps reared with probiotics (Maeda, 1994; Austin *et al.*, 1995; Azad *et al.*, 2005). These beneficial microbes can be effectively used against the pathogenic *Vibrio* species, which is a major

problem in shrimp hatcheries (Moriarty, 1998; Rengpipat *et al.*, 1998; Otta *et al.*, 2014). Apparently, probiotics serve as immunostimulants as well as immunomodulators which elicit the non-specific immunity in shrimps to fight infections and diseases. The mechanism by which probiotic bacteria enhance immunity of fish is still not completely elucidated to date though there are several studies (Irianto and Austin, 2002; Panigrahi *et al.*, 2009, 2011).

Owing to the varied potential of probiotics, the present study was aimed to investigate the effect of selected probiotics (commercial, conventional and isolated bacterial cultures) on the growth performance and immunomodulation of pacific white-legged shrimp, *Penaeus vannamei*.

Materials and methods

Probiotics sourcing and mass production

The experiment was conducted using a commercially available probiotics (Cp) and 6 probiotics prepared from pure stains *i.e., Bacillus subtilis* (MTCC 2756), *Lactobacillus rhamnosus* (ATCC 53103), *Lactobacillus casei* (ATCC 335), *Saccharomyces cerevisiae* (IAM 14383T), *Saccharomyces boulradi* and *Enterococcus* sp. (MTCC 10646), Pure stains of *S. boulradi* were collected fron known sources from central labs including (ICAR-CIBA). For analysing the viability of the strains, the bacteria were cultured at 30°C for 24 to 36 h in specific media *viz.,* tryptic soy agar (Merck, Darmstadt, Germany) for *Bacillus subtilis*; Enterococcus differential agar base (TITG Agar Base, HiMedia, USA) for *Enterococcus* sp.; MRS agar (Merck, Darmstadt, Germany) for *Lactobacillus casei* and *Lactobacillus rhamnosus;* Sabouraud dextrose agar (HiMedia, USA) for *Saccharomyces cerevisiae* and *Saccharomyces boulardii* and subsequently preserved in glycerin at -80°C for further use. Later identification of these strains were confirmed using biochemical and molecular tools using 16 S ribosomal RNA. These potential probiotic strains were mass produced by Mystical Biotech Pvt. Ltd. Hoskote, Bangalore, India for further experimentation.

Probiotic feed preparation

Shrimp feed was formulated at ICAR-Central Institute of Brackishwater Aquaculture (ICAR-CIBA), Chennai as per Castex *et al.* (2006). Seven probiotic feeds were prepared by top coating of the respective probiotics (at a concentration of 5 x 10⁹ CFU g⁻¹) with every kilogram of feed using guar gum. The final concentration of probiotics in feed was maintained @ 5 x 10⁶ CFU g⁻¹ of respective probiotics kg⁻¹ of feed. The pellet feeds were prepared at the institute feed mill of ICAR-CIBA, Chennai and subsequently top coated with the respective probiotics to prepare the experimental feeds. The concentration of probionts in the feed was confirmed

by spread plate method in the respective media as described earlier.

Experimental design

P. vannamei post-larvae (PL III) were purchased from Vaisakhi Shrimp Hatchery at Marakkanam, Tamil Nadu. The experiment was conducted at the Muttukadu Experimental Station of ICAR-CIBA, Chennai, in 100 l fibre-reinforced plastic (FRP) tanks (50 cm x 70 cm x 35 cm), with each treatment in triplicates. Each tank was stocked with 50 PL of *P. vannamei* having mean initial weight of 0.08 g. Water was exchanged (20%) once in three days and water quality parameters were monitored regularly. The seawater used for rearing was ideal for *P. vannamei* culture with salinity of 33 ± 1.0 ppt. The aeration was optimum ensuring dissolved oxygen (DO) levels in the range of 7 to 8 ppm. The experiment was conducted for a period of 75 days during which the PLs were fed with probiotics supplemented diet @4% of the body weight twice a day at 08 00 and 18 00 hrs.

Assessment of physico-chemical parameters

Seawater (33±1.0 ppt) was used for rearing shrimps. Water quality was checked on weekly basis. Water parameters such as temperature (mercury thermometer), pH (pH-Scan-Eutech instruments, Singapore), total ammonia nitrogen (TAN) (Phenol hypochlorite method), NO_2-N, NO_3-N and dissolved oxygen (DO) were analysed following APHA (1998).

Assessment of growth performance

The growth performance of shrimps was recorded once in 15 days by measuring the length and weight followed by estimation of survival rate, specific growth rate (SGR) and average daily growth (ADG) using the following formulae:

$$\text{Weight gain (\%)} = \frac{\text{Final weight (g)-Initial weight (g)/Initial weight}}{\text{(mg)}}$$

$$\text{Length gain (\%)} = \frac{\text{Final length (mm)-Initial length (mm)/}}{\text{Initial length (mm)} \times 100}$$

$$\text{SGR (\%)} = \frac{\text{(In final weight-In initial weight)/Days of culture}}{\times 100}$$

$$\text{ADG (g day}^{-1}) = \frac{\text{Final weight (mg)-Initial weight (mg)/}}{\text{Experimental duration (days)}}$$

$$\text{Survival (\%)} = \frac{\text{Shrimp no. at the end of experiment /Shrimp no.}}{\text{at the beginning of experiment} \times 100}$$

Assessment of microbial load

The total heterotrophic bacteria were determined in the shrimp rearing water by counting the colonies which grew on plates of Zobell Marine Agar (ZMA) (Hi-Media) with 1% of NaCl (Jorgensen *et al.*, 1993). Before plating each sample

onto agar medium, serial dilutions were made in physiological saline (0.9% NaCl) solution (Sohier and Bianchi, 1985). The total *Vibrio* count in water samples were enumerated using thiosulphate citrate bile salt sucrose (TCBS) agar (Hi-Media) by spread plate technique as previously described (Harris *et al.*, 1996). The bacterial counts were expressed in colony forming units per ml of water (CFU ml^{-1}) (Smith, 1998).

Collection and analysis of haemolymph

Physiological saline was prepared by dissolving NaCl (340 mm), KCl (13 mm), MgSO$_4$ (11 mm), MgCl$_2$ (10 mm), NaH$_2$PO$_4$ (0.3 mm) and glucose (1.6 mm) in 100 ml distilled water and pH was adjusted to 7.8 using NaHCO$_3$ (Hi-Media). Anticoagulant saline (ACS) was prepared by adding 3 mg cysteine in 5 ml of physiological saline. Hemolymph samples from probiotics treated and control shrimps were withdrawn from heart using 21 G needle attached to a 2 ml sterile polypropylene syringe containing 1 ml of ice-cold cysteine anticoagulant saline. After hemolymph collection, the syringe was withdrawn from the animal and shaken gently to assist the rapid mixing of hemolymph and ACS.

Total haemocyte count

Haemolymph (100 µl) was withdrawn from the ventral sinus of the first abdominal segment into a syringe containing anti-coagulant saline (900 µl) and transferred to an eppendorf tube for total haemocyte count (THC). The THC was measured according to modified method from Soderhall and Smith (1983). Briefly, 10 µl of haemolymph collected from each individual was introduced into an improved Neubauer haemocytometer and the number of haemocytes was determined microscopically.

Phenoloxidase activity assay

Haemolymph samples were collected from control and probiotic treated shrimps by cardiac puncture using a sterile syringe. The haemolymph samples thus collected were transferred to microcentrifuge tubes held on ice and allowed to clot for 30 min at room temperature (28±2˚C). The clot was disturbed using a clean glass rod and then centrifuged at 1500 rpm for 7 min. The clear supernatant (serum) thus collected was used for phenoloxidase activity as per Smith and Soderhall (1983).

Statistical analysis

All results are presented as the average (mean±SD) of at least three independent experiments. Student's t-test, analysis of variance (ANOVA) and the Duncan's multiple range tests were used for statistical analyses of the data (p<0.05) performed using SPSS for Windows version 20.0.

Results and discussion

Reports on the efficacy of probiotics on the growth and survival of the domesticated shrimp, *P. vannamei*

are inadequate. In this context, the present study was conducted to investigate the effect of six different probiotic bacteria and a commercial preparation on the growth performance, survival and immunomodulation of *P. vannamei*. Application of *B. subtilis* as probiotic has brought very promising results for shrimp aquaculture. This is a nonpathogenic Gram positive spore-forming bacterium which has been used to improve the growth performance, as well as shrimp health and disease management in shrimp farming (Balcazar *et al.*, 2007; Keysami *et al.,* 2012). In addition, it is well documented that *Bacillus* sp. are able to produce a wide range of extracellular substances and antimicrobial peptides against a variety of microorganisms (Perez *et al.*, 1993; Korenblum *et al.*, 2005). *S. cerevisiae* is a potential supplement in shrimp feeds (Gabriel Aguirre-Guzma´n *et al.*, 2002) and has been reported to have beneficial effects in the shrimp growth performance, immunity and ability to improve water quality. The results of the present study indicated a significant (p<0.05) improvement in growth in terms of weight and length in all the probiotic fed groups (Table 1). An increase of 50-95% growth was observed in probiotic fed groups with maximum increase in *S. cerevisiae* (8.05±0.21 g), followed by *B. subtilis* (7.65±0.21 g) and other probiotic treated groups (Sb = 7.00±0.28, Lr = 6.30±0.14, Lc = 6.2±0.28 and Ent.= 5.1±0.14 g) whereas the control showed lower growth of 4.85±0.49 g (Fig. 1).
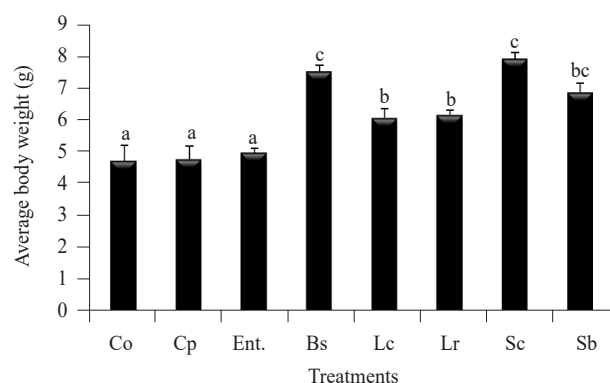

Fig. 1. Average body weight of *P. vannamei* fed probiotic incorporated and control diets. Data expressed as mean ± S.D. Groups with different superscript letters differ significantly.

A similar trend was also observed in the average body length (ABL) of shrimps fed with different probiotics (Table 1). Several authors reported better growth performances in probiotic treated shrimps when compared to control groups (Intriago *et al.*, 1998; Rengpipat *et al.*, 1998; Wang *et al.*, 2007).

The survival rate of *P. vannamei*, treated with probiotic *S. cerevisiae* was found to be the highest (65.5±0.7%), followed by other groups such as Bs (59.0±1.4%), Sb (56.5±0.7%), Lr (54.5±0.7%), Lc (52.5±2.1%),

Table 1. Growth performance of *P. vannamei* fed probiotic incorporated and control diets for a period of 75 days

Treatments	Initial weight (g)	ABW (g)	Weight improvement (%)	Initial length (mm)	ABL (mm)	Length improvement (%)	SGR (%)	ADG (g day^{-1})
CO	0.08±0.00[a]	4.85±0.49[a]	-	15.7±0.06[a]	63.0±1.41[a]	-	2.08±0.14[a]	0.062±0.004[a]
Cp	0.08±0.01[a]	4.90±0.42[a]	1.0±0.52[a]	15.6±0.06[a]	64.0±1.23[a]	1.5±0.06[a]	2.09±0.12[a]	0.064±0.002[a]
Ent	0.08±0.00[a]	5.10±0.14[a]	5.2±1.2[a]	15.8±0.08[a]	65.0±1.41[a]	3.1±0.05[a]	2.15±0.04[a]	0.065±0.004[a]
Bs	0.08±0.01[a]	7.65±0.21[c]	57.7±2.7[c]	15.6±0.09[a]	97.5±2.12[c]	54.4±0.47[bc]	2.70±0.04[b]	0.104±0.002[c]
Lc	0.08±0.00[a]	6.20±0.28[b]	27.8±1.2[b]	15.8±0.04[a]	72.5±0.71[b]	15.0±0.06[b]	2.44±0.03[ab]	0.082±0.001[b]
Lr	0.08±0.00[a]	6.30±0.14[b]	29.9±1.2[b]	15.5±0.00[a]	75.5±0.71[b]	19.8±0.03[b]	2.41±0.06[ab]	0.083±0.001[b]
Sc	0.08±0.00[a]	8.05±0.21[c]	66.0±3.2[c]	15.7±0.08[a]	103.5±0.71[c]	64.2±0.06[c]	2.77±0.04[b]	0.106±0.002[c]
Sb	0.08±0.00[a]	7.00±0.28[bc]	44.3±1.2[bc]	15.7±0.00[a]	92.5±0.71[bc]	46.8±0.09[ab]	2.58±0.05[ab]	0.092±0.002[bc]

Data expressed as mean ± S.D. Means with different superscript letters in the column differ significantly (p<0.05)
CO : Control, Cp : Commercial probiotic, Ent : *Enterococcus* sp*.,* Bs : *Bacillus subtilis,* Lc : *Lactobacillus casei,* Lr : *Lactobacillus rhamnosus*, Sc : *Saccharomyces cerevisiae*, Sb : *Saccharomyces boulardii.* Control : Without addition of probiotics, SGR : Specific growth rate, ADG : Average daily growth
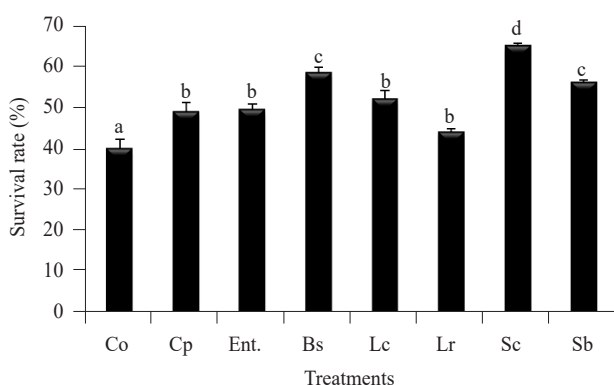

Fig. 2. Average survival % of juvenile Pacific white shrimp reared with different probiotic feeds. Data expressed as mean±S.D. Groups with different superscript letters differs significantly

Ent (49.9±1.4%) and Cp (49.5±2.1%) compared to control (40.5±2.1% (Fig. 2).

Moriarty (1998) has reported increased shrimp survival in the pond water inoculated with *Bacillus* species. This was attributed to the water quality improvement by the bacterial inoculations, due to degradation of organic matter. In accordance with our results, there are also reports of enhancement in shrimp performance when probiotics are used as feed additives (Wang *et al.*, 2005; De Souza *et al.*, 2012; Silva *et al.*, 2012). The SGR of *P. vannamei* was observed to be maximum in *S. cerevisiae* treated group (10.6±0.03%) followed by Bs>Lc>Lr>Sb>Ent>Cp (Table 1). Average daily growth (ADG) was high in *S. cerevisiae* (0.106±0.002 g) and *B. subtilis* (0.104 ± 0.002 g) followed by other strains such as Sb (0.092 ± 0.002 g), Lc (0.083 ± 0.001 g), Lr (0.082 ± 0.001 g), Ent (0.065 ± 0.004) and Cp (0.064 ± 0.002 g) whereas in control it was observed to be the lowest (0.062 ± 0.004 g). Similar to our findings, several studies have demonstrated the beneficial effects of probiotics on the growth performance in shrimp (Wang *et al.*, 2007; Shen *et al.*, 2010; Liu *et al.*, 2009).

Further, non-specific immune parameters in terms of THC and proPO showed strain specific enhancement in probiotic treated groups (Smith and Soderhall, 1983). Haemocytes play vital role in the immune response in crustaceans. Probiotic *S. cerevisiae* were found to trigger the haemocytes and in turn the immune system in the test animals (Ratcliffe and Rowley, 1979; Soderhall *et al.*, 1984). The THC was found to be high in the *S. cerevisiae* (12.4±0.8) x 10^6 cells ml^{-1}) and other probiotic treated groups of Pacific white shrimp (Bs = 11.2±0.8, Sb = 9.1±0.5, Lc = 8.8±0.4, Lr = 8.8±0.4, Ent. = 7.1±0.8 and Cp = 6.2±0.8 X 10^6 cells ml^{-1}) compared to that observed in control (5.2±0.9 X 10^6 cells ml^{-1}) (p<0.05) (Fig. 3).
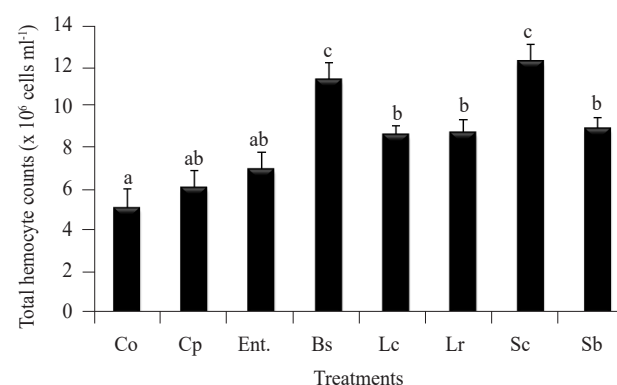

Fig. 3. Average total haemocyte count (x 10^6 cells ml^{-1}) of juvenile Pacific white shrimp sub adult stage (n=6) reared with different probiotic feeds. Data expressed as mean±S.D. Groups with different superscript letters differ significantly.

The proPO activating system has a fundamental role in organising the innate immune response in invertebrates (Soderhall and Cerenius, 1998). Enhanced phenoloxidase activity in probiotic treated groups clearly demonstrates shrimps' ability to respond to external challenges (Lee and Soderhall, 2002). The pro PO system plays a critical role in

host defensive reactions, which could be activated by some microbial lipopolysaccharides and β-1,3-glucan to generate phenoloxidase (PO), an important enzyme in the process of melanisation (Gai *et al.*, 2008; Figueroa-Pizano *et al.*, 2014). The transcript levels of proPO generally corresponded to the activity monitored for the same enzyme (Gai *et al.*, 2008). Furthermore, proPO activity was found to be significantly higher ($p<0.05$) in the *S. cerevisiae* probiotic fed (0.132 ± 0.001U min^{-1} mg protein^{-1}) group of *P. vannamei* and other probiotic groups (Bs = 0.130 ± 0.002, Lr = 0.125 ± 0.005, Lc = 0.124 ± 0.005, Sb = 0.110 ± 0.005, Ent. = 0.103 ± 0.002 and Cp = 0.089 ± 0.004 U min.$^{-1}$ mg protein^{-1}) when compared to control (0.079 ± 0.001 U min.$^{-1}$ mg protein^{-1}) (Fig. 4). Although several probiotic bacteria were reported to exert beneficial effects on PO activity in shrimp (Rengpipat *et al.*, 2000; Tseng *et al.*, 2009; Sapcharoen and Rengpipat, 2013), a few of them like *B. subtilis* BP11 did not show any siginifical effect on PO activity (Sapcharoen and Rengpipat, 2013).
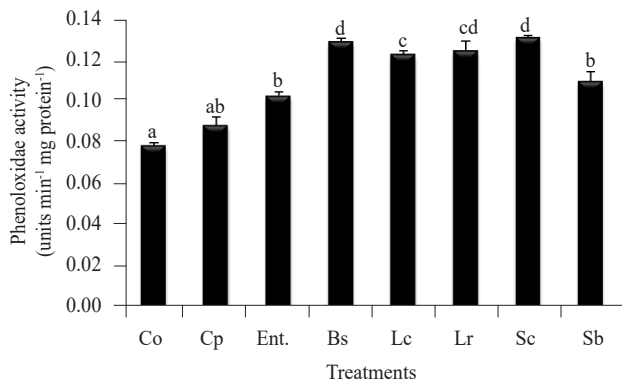

Fig. 4. Phenoloxidase activity (units min^{-1} mg protein^{-1}) in juvenile Pacific white shrimp reared with different probiotic diets and control. Data expressed as mean ± S.D. Groups with different superscript letters differ significantly.

Additionally, the probiotic treated groups also showed a drastic reduction in *Vibrio* count (Fig. 5a) and an increase in heterotrophic bacteria count (Fig. 5b). Further, the level of ammonia in rearing water decreased in the probiotic treated groups such as *S. cerevisiae* ($0.0.106\pm0.0$ ppm), Bs (0.108 ± 0.0 ppm), Lc (0.114 ± 0.0 ppm), Lr (0.121 ± 0.0 ppm), Ent (0.124 ± 0.0 ppm) and Sb (0.126 ± 0.0 ppm). Similarly, nitrate and nitrite levels were found decreased in the probiotic treated groups *i.e. S. cerevisiae* (0.018 ± 0.001 ppm) whereas in the control group, it was 0.089 ± 0.005 ppm (Table 2). There was a decrease in nitrite and nitrate concentration for probiotic treated group. *S. cerevisiae* treated group (0.018 ± 0.001 ppm) showed the lowest nitrite and nitrate levels compared to control (0.089 ± 0.005 ppm) and other treatments (Table 2).

These parameters provide a favourable environment for shrimp growth and survival. Water quality parameters such as temperature, pH, salinity, dissolved oxygen and phosphate ranged from 27 to 28°C, 8.17 to 8.27, 32.0 to 33.0 ppt, 8.13 to 8.53 ppm and 0.070 to 0.082 ppm respectively in probiotic fed group and no significant ($p<0.05$) difference were found compared to that of the control.

Wang *et al.* (2005) reported reduction of the nitrogen and phosphorous levels in *P. vannamei* ponds using the same microorganisms. However, the TAN, nitrite-N and nitrate-N levels were decreased in the probiotic treated tanks (Table 2). Our results suggest the plausible role of probiotics in improving the water quality in aquaculture ponds. Probiotic products used in aquaculture ponds were reported to give better overall growth and water quality (Rengpipat *et al.*, 1998; Das *et al.*, 2006; Zhu *et al.*, 2009). The degradation of organic matter by probiotics like *Bacillus* sp. might help to improve water quality (Gatesoupe, 1999).
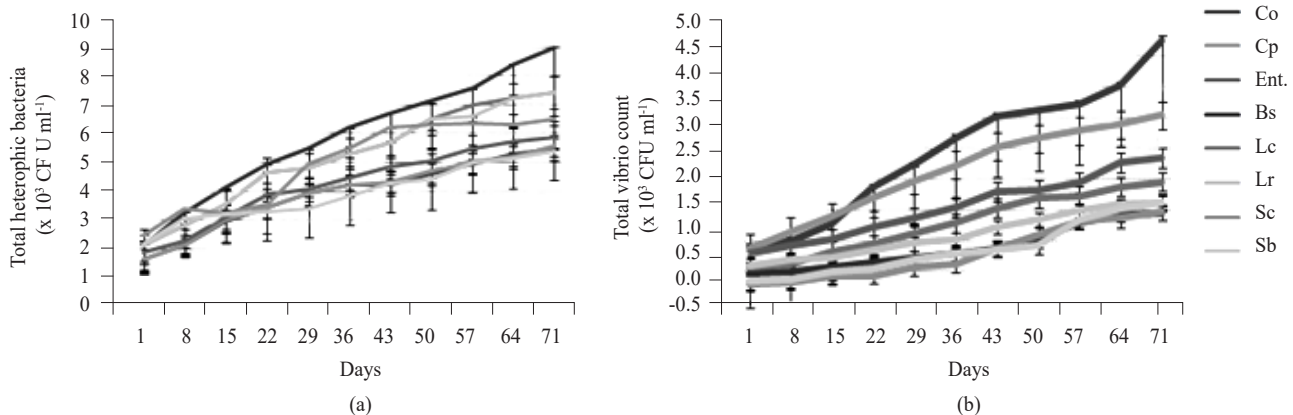

Fig. 5. Reduction of vibrio count (a) in the probiotic fed group when compared to the control and increase in the total bacterial count (b) in the probiotic fed groups

Table 2. Physico-chemical parameters of water in the experimental tanks of *P. vannamei* fed probiotic incorporated and control diets

Treatments	Temperature (^{0}C)	pH	Salinity (ppt)	TAN (ppm)	NO_2 (ppm)	NO_3 (ppm)	PO_4 (ppm)	DO (ppm)
CO	28.0±0.0[a]	8.33±0.02[a]	33.0±0.0[a]	0.500±0.0[a]	0.044±0.0[a]	0.071±0.005[a]	0.084±0.003[a]	8.74±0.29[a]
Cp	28.0±0.0[a]	8.27±0.01[ab]	33.0±0.0[a]	0.247±0.0[b]	0.018±0.0[b]	0.021±0.004[b]	0.082±0.002[a]	8.74±0.29[a]
Ent	28.0±0.0[a]	8.24±0.04[ab]	33.0±0.0[a]	0.124±0.0[ab]	0.020±0.0[a]	0.019±0.001[b]	0.080±0.002[a]	8.74±0.29[a]
Bs	28.0±0.0[a]	8.22±0.01[ab]	33.0±0.0[a]	0.108±0.0[c]	0.011±0.0[b]	0.005±0.001[c]	0.070±0.002[a]	8.13±1.15[a]
Lc	28.0±0.0[a]	8.18±0.01[b]	33.0±0.0[a]	0.114±0.0[c]	0.018±0.0[b]	0.015±0.001[b]	0.077±0.001[a]	8.53±0.00[a]
Lr	28.0±0.0[a]	8.17±0.01[b]	33.0±0.0[a]	0.121±0.0[ab]	0.018±0.0[b]	0.015±0.004[b]	0.078±0.001[a]	8.53±0.00[a]
Sc	28.0±0.0[a]	8.19±0.01[b]	33.0±0.0[a]	0.106±0.0[c]	0.007±0.0[c]	0.003±0.001[c]	0.074±0.002[a]	8.33±0.29[a]
Sb	28.0±0.0[a]	8.18±0.28[b]	33.0±0.0[a]	0.126±5.0[ab]	0.021±0.0[b]	0.010±0.001[b]	0.077±0.001[a]	8.33±0.29[a]

Values are mean of triplicate groups and presented as mean ± SE. Values with different superscripts in the same row are significantly different ($p<0.05$).

Moriarty (1998) found that the use of probiotics could prevent luminescent *Vibrio* infection by either lowering or completely eliminating luminous vibrios in pond water and sediment. Devaraja *et al.* (2002) reported that *B. subtilis* and *S. cerevisiae* treated groups had a significantly higher concentration of total heterotrophic bacteria when compared to other treatments. The present study showed that the microbial loads were significantly altered in the treated groups and the heterotrophic bacterial (THB) counts in the rearing water were also significantly ($p<0.05$) higher in treated groups than the control indicating their dominance in the culture system (Fig. 5a). Interestingly, the *Vibrio* counts were drastically declined in the all the probiotic fed groups indicating the plausible dominance of THB over the pathogenic *Vibrio* spp. (Fig. 5b).

In conclusion, the present study highlights the importance of various probiotics in improving the growth and survivability of *P. vannamei* post-larvae. Better performance was observed in groups fed with probiotic incorporated diets like *Saccharomyces cerevisiae, B. subtilis, S. boulardii, L. casei* and *L. rhamnosus* when compared to control. Probiotic treatment has also shown to elicit the cellular and humoral immunity in the tested animals, thus explaining the mechanism of action of these probionts. Hence, we propose that the probiotics particularly *S. cerevisiae* and *B. subtilis* have the potential to be used in shrimp culture owing to their beneficial effects. Further study is warranted to clearly understand the target pathways and the mechanism of action of probiotics in shrimps.

Acknowledgements

References

APHA/AWWA/WPCF 1998. *Standard methods for the examination of water and waste water,* 20th edn., American Public Health Association, Washington, DC, USA, 1325 pp.

Austin, B., Stuckey, K. F., Robertson, P. A., Efendi, I. and Griffith, D. R. W. 1995. A probiotic strain of *Vibrio alginolyticus* effective in reducing diseases caused by *Aeromonas salmonicida, Vibrio anguillarum* and *Vibrio ordalii. J. Fish Dis.*, 18: 93-96.

Azad, I. S., Panigrahi, A., Gopal, C., Paulpandi, S., Mahima, C. and Ravichandran, P. 2005. Routes of immunostimulation *vis-a vis* survival and growth of *Penaeus monodon* (Fabricius) post-larvae. *Aquaculture*, 248: 227-234.

Balcazar, J. L. and Rojas-Luna, T. 2007. Inhibitory activity of probiotic *Bacillus subtilis* UTM126 against vibrio species confers protection against vibriosis in juvenile shrimp (*Litopenaeus vannamei*). *Curr. Microbiol.*, 55: 409-412.

Castex, M., Chim, L., Wabete, N., Lemaire, P. and Usache, V. 2006. Feeding evaluation of probiotic bacteria *Pediococcus acidilactici* (Bactocell®) in subadult shrimp *Litopenaeus stylirostris*: microbial, nutritional and zootechnical aspects. In: *Book of Abstract, WAS Annual Meeting,* Florenze, Italia.

Das, S., Lyla, P. S. and Khan, S. A. 2006. Application of *Streptomyces* as a probiotic in the laboratory culture of *Penaeus monodon* (Fabricius). *Isr. J. Aquac. Bamidgeh*, 58: 198-204.

De Souza, D. M., Suita, S. M., Leite, F. P. L., Romano, L. A., Wasielesky, W. and Ballester, E. L. C. 2012. The use of probiotics during the nursery rearing of the pink shrimp *Farfantepenaeus brasiliensis* (Latreille, 1817) in a zero exchange system. *J. Aquac. Res.*, 43: 1828-1837.

Devajara, T. N., Yusoff, F. and Shariff, M. 2002. Changes in bacterial populations and shrimp production in ponds treated with commercial microbial products. *Aquaculture*, 206(3-4): 254-256.

Guzman, G. A., Marie, D. R. and Suarez, L. E. C. 2002. Survival of agglomerated *Saccharomyces cerevisiae* in pelleted shrimp feeds. *Aquaculture*, 208: 125- 135.

Gai, Y. C., Zhao, J. M., Song, L. S., Li, C. H., Zheng, P. L., Qiu, L. M. and Ni, D. J. 2008. A prophenoloxidase from the Chinese mitten crab *Eriocheir sinensis*: gene cloning, expression and activity analysis. *Fish Shellfish Immunol.*, 24: 156-167.

Gatesoupe, F. J. 1999. The use of probiotics in aquaculture. *Aquaculture*, 180: 147-165.

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

Harris, L., Owens, L. and Smith, S. 1996. A selective and differential medium for *V. harveyi. Appl. Environ. Microbiol*., 62: 3548-3550.

Intriago, P., Kraussand, E. and Barniol, R. 1998. The use of yeast and fungi as probiotics in *Penaeus vannamei* larviculture. In: *Book of Abstracts*, *International Triennial Conference and Exposition,* World Aquaculture Society, National Shellfish Association and American Fisheries Society, Las Vegas 15-19 February, 1998, Nevada, USA, 263 pp.

Irianto, A. and Austin, B. 2002. Probiotics in aquaculture. *J. Fish Dis.,* 25: 633-642.

Itami, T., Takahashi, Y., Yoneoka, K. and Yan, Y. 1991. Survival of larval giant tiger prawns *Penaeus monodon* after addition of killed *Vibrio* cells to a microencapsulated diet. *J. Aquat. Anim. Health*, 3: 151-152.

Itami, T., Takahashi, Y. and Nakamura, Y. 1989. Efficacy of vaccination against vibriosis in cultured kuruma prawns *Penaeus japonicus*. *J. Aquat. Anim. Health*, 1: 238-242.

Jorgenson, N. O. G., Kroer, N., Coffin, R. B., Yang X. H. and Lee, C. 1993. Dissolved free amino acids, combined amino acids and DNA sources of carbon and nitrogen to marine bacteria. *Mar. Ecol. Prog. Ser*., 98: 135-148.

Karunasagar, I., Pai, R., Malathi, G. R. and Karunasagar, I. 1994. Mass mortality of *Penaeus monodon* larvae due to antibiotic-resistant *Vibrio harveyi* infection. *Aquaculture*, 128: 203-209.

Karunasagar, I., Shivu, M. M., Girisha, S. K., Krohne, G. and Karunasagar, I. 2007. Biocontrol of pathogens in shrimp hatcheries using bacteriophages. *Aquaculture*, 286: 288-292.

Keysami, M., Mohammadpour, M. and Saad, C. 2012. Probiotic activity of *Bacillus subtilis* in juvenile freshwater prawn, *Macrobrachium rosenbergii* (de Man) at different methods of administration to the feed. *Aquac. Int*., 20: 499-511.

Korenblum, E., von Der Weid, I., Santos, A. L. S., Rosado, A. S., Sebastián, G. V. and Coutinho, C. M. L. M. 2005. Production of antimicrobial substances by *Bacillus subtilis* LFE-1, *B. firmus* H2O-1 and *B. licheniformis* T6-5 isolated from an oil reservoir in Brazil. *J. Appl. Microbiol*., 98: 667-675.

Lee, S. and Soderhall, K. 2002. Early events in crustacean innate immunity. *Fish Shellfish Immunol*., 12: 421-437.

Liu, C. H., Chiu, C. S., Ho, P. L. and Wang, S. W. 2009. Improvement in the growth performance of white shrimp, *Litopenaeus vannamei*, by a protease-producing probiotic, *Bacillus subtilis* E20, from natto. *J. Appl. Microbiol.*, 107: 1031-1041.

Maeda, M. 1994. Biocontrol of the larval rearing biotope in aquaculture. *Bull. Natl. Res. Inst. Aquac., Supplement*, 1: 71-74.

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

Otta, S. K., Arulraj, R., Praveena, E. P., Manivel, R., Panigrahi, A., Bhuvaneswari, T., Ravichandran, P., Jithendran, K. P. and Ponniah, A. G. 2014. Association of dual viral infection with mortality of Pacific white shrimp (*Litopenaeus vannamei*) in culture ponds in India. *Indian J. Virol.*, 25(1): 63-68.

Panigrahi, A., Kiron, V., Puangkaew, J., Kobayashi, T., Satoh, S. and Sugita, H. 2005. The viability of probiotic bacteria as a factor influencing the immune response in rainbow trout *Oncorhynchus mykiss*. *Aquaculture*, 243: 241-254.

Panigrahi, A., Kiron, V., Satoh, S., Hirono, I., Kobayashi, T., Aoki, T., Puangkaew, J. and Sugita, H. 2007. Immunomodulation and expression of immune related genes in rainbow trout *Oncorhynchus mykiss* fed probiotic-supplemented diets. *Dev. Comp. Immunol*., 31: 372-382.

Panigrahi, A., Azad, I. S., Das, B. K., Dandpat, J., Das, G., Behera, S. and Mishra, S. S. 2009. Probiotic induced immunomodulation: investigation into the cellular and molecular mechanism involved. *Res. J. Biotechnol.*, 4 (3): 7-13.

Panigrahi, A., Viswanath, K. and Satoh, S. 2011. Real-time quantification of the immune gene expression in rainbow trout fed different forms of probiotic bacteria *Lactobacillus rhamnosus. Aquac. Res.,* 42: 906-917.

Perez, C., Suarez, C. and Castro, G. 1993. Antimicrobial activity determined in strains of *Bacillus circulans* cluster. *Folia Microbiol.*, 38: 25-28.

Ratcliffe, N. A. and Rowley, A. F. 1979. Comparative synopsis of the structure and function of the blood cells of insects and other invertebrates. *Dev. Comp. Immunol*., 3: 189-221.

Rengpipat, S., Phianphak, W., Piyatiratitivorkaul, S. and Menasveta, P. 1998. Effects of probiotic bacterium in black tiger shrimp *Penaeus monodon* survival and growth. *Aquaculture*, 167: 301-313.

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

Sapcharoen, P. and Rengpipat, S. 2013. Effects of the probiotic *Bacillus subtilis* (BP11 and BS11) on the growth and survival of Pacific white shrimp, *Litopenaeus vannamei. Aquac. Nutr*., 19: 946-954.

Shen, W. Y., Fu, L. L., Li, W. F. and Zhu, Y. R. 2010. Effect of dietary supplementation with *Bacillus subtilis* on the growth, performance, immune response and antioxidant activities of the shrimp (*Litopenaeus vannamei*). *Aquac. Res*., 41: 1691-1698.

Smith, V. J., Soderhall, K. and Hamilton, M. 1984. β-1,3-glucan induced cellular defense reaction in the shore crab, *Carcinus maenas*. *Comp. Biochem. Physiol*., 77A: 636-639.

Smith, P. T. 1998. Effect of removing accumulated sediments on the bacteriology of ponds used to culture *Penaeus monodon*. *Asian Fish. Sci*., 10: 355-370.

Silva, E. F., Sores, M. A., Calazans, N. F., Vogeley, J. L., do Valle, B. C., Soares, R. and Peixoto, S. 2012. Effect of probiotic (*Bacillus* spp.) addition during larvae and post-larvae culture of the white shrimp *Litopenaeus vannamei. Aquac. Res.*, 44: 13-21.

Soderhall, K. and Smith, V. J. 1983. Separation of hemocyte populations of *Carcinus maenas* and other marine decapods, and prophenoloxidase distribution. *Dev. Comp. Immunol.*, 7: 229-239.

Soderhall, K., Vey, A. and Ramstedt, M. 1984. Hemocyte lysate enhancement of fungal spore encapsulation by crayfish hemocytes. *Dev. Comp. Immunol.*, 8: 23-29.

Soderhall, K. and Cerenius, L. 1998. Role of the prophenoloxidase-activating system in invertebrate immunity. *Curr. Opin. Immunol.*, 10: 23-28.

Sohier, L. P. and Bianchi, M. A. G. 1985. Development of a heterotrophic bacterial community within a closed prawn aquaculture system. *Microbial Ecol.*, 11: 353-369.

Son, V. M., Chang, C. C., Wu, M. C., Guu, Y. K., Chiu, C. H. and Cheng, W. 2009. Dietary administration of the probiotic, *Lactobacillus plantarum*, enhanced the growth, innate immune responses and disease resistance of the grouper *Epinephelus coioides. Fish Shellfish Immunol.*, 26: 691-698.

Tseng, D. Y., Ho, P. L., Huang, S. Y., Cheng, S. C., Shiu, Y. L., Chiuand, C. S. and Liu, C. H. 2009. Enhancement of immunity and disease resistance in the white shrimp, *Litopenaeus vannamei*, by the probiotic, *Bacillus subtilis* E20. *Fish Shellfish Immunol.*, 26: 339-344.

Wang, Y. B., Xu, Z. R. and Xia, M. S. 2005. The effectiveness of commercial probiotics in Northern white shrimp (*Penaeus vannamei* L.) ponds. *Fish. Sci.*, 71: 1034-1039.

Wang, Y. B. and Han, J. Z. 2007. The role of probiotic cell wall hydrophobicity in bioremediation of aquaculture. *Aquaculture*, 269: 349-354.

Zhu, C. H., Zhu, G. Q., Musa, H. H., Seri, H. I. and Wu, S. L. 2009. The potential benefits of probiotics in animal production and health. *J. Anim. Vet. Adv.*, 8: 313-321.