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Research Article
Performance Evaluation of Two Probiotic Species, on the Growth, Body Composition and Immune Expression in *Penaeus monodon*

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Abstract

Background and Objective: The probiotics used in marine shrimp farms are based predominantly on the terrestrial probiotic and their modes of action are not established well. This study was an attempt to evaluate the performance efficiency of two putative probiotic strains, on the growth, survival, biochemical enhancement, immune expression and disease resistance in *Penaeus monodon*. **Materials and Methods:** The three feed trial groups in the study composed of (1) *B. coagulans* (BSCB-2), (2) *B. firmus* (BSCB-13) and (3) The mixed bacterial diet group comprising equal proportion of the two bacterial strains. The selected strains were incorporated in specific quantities in the compounds shrimp feed and were fed to *P. monodon* for 15 days, after a pathogen challenge with *Vibrio alginolyticus*. Growth, biochemical and immune parameters were studied. Statistical analysis was performed using SPSS version 10.5. **Results:** Mean weight gain (g), mean length gain (cm), Food Conversion Ratio (FCR) and Specific Growth Rate (SGR) significantly increased ($p<0.05$) in the combined bacterial diet group compared to other groups. The biochemical constituents such as protein and glycogen were also significantly higher ($p<0.05$) in bacterial diet group, while lipid variation was insignificant. Total Haemocyte Count (THC) was significantly higher in *B. firmus* fed group (1584±6.0). Gradual decrease in THC was observed generally after infection. Maximum reduction was observed in control animals (561±5.0). Prophenol oxidase activity was higher in *B. coagulans* group (7.3±0.2 U min^{-1} mg^{-1} of protein), while decrease in Prophenol oxidase activity was observed in control animals (1.7±0.1 U min^{-1} mg^{-1} of protein). The NBT activity significantly increased ($p<0.05$) in *B. firmus* (4.21±0.2) supplemented group. A gradual decrease in nitroblue tetrazolium (NBT) activity was observed in control animal group (0.88±0.1). Bacterial clearance was enumerated in the haemolyph from the time of *Vibrio* injection. There was an initial spurt of *Vibrios* when cultured in the selective medium TCBS for all the three treatments. A gradual decrease in *Vibrio* count was observed after 24 h duration. **Conclusion:** It is concluded that oral administration of probiotics led to their adherence in shrimp digestive tract. Also, Probiotic supplementation increased the resistance of shrimps to *V. alginolyticus* infection and brought about increased survival.

Key words: *Bacillus coagulans, Bacillus firmus*, *Penaeus monodon*, immune parameters, probiotics, *Vibrio alginolyticus*

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

There exists a growing demand of animal proteins for human consumption and the sources of animal proteins are mainly terrestrial and aquatic animals[1]. World aquaculture has grown tremendously during the last 50 years, yet aquaculture is facing serious pitfalls because of recent disease breakouts[2]. Frequent diseases added with deterioration of environment leads to serious economic losses[3]. Bacterial infection is one of the major causes of diseases in aquaculture[4]. With the fast increase of shrimp culture, serious disease outbreaks has become an ever-increasing problem[5]. *Vibrio* is the most significant bacterial spp. causing high mortality among shrimps worldwide[6]. *Vibrio* disease is described as 'vibriosis' or 'bacterial disease', 'penaeid bacterial septicaemia', 'penaeid vibriosis', 'luminescent vibriosis' or 'red leg disease'[7]. In the last few decades, antibiotics were used as traditional strategy for managing fish and prawn diseases and also for maintaining feed quality[8]. As the regular use of antibiotics and chemicals as preventive as well as curative measures for diseases leads to the emergence of drug resistant bacteria and creates harmful effect on the environment[9], finding alternates for antibiotics and chemicals are the need of the hour to control vibriosis[10].

Probiotics as live microbial feed supplement beneficially affect the host animal by improving its intestinal microbial balance[11]. The use of probiotics in aquaculture is increasing over the years due to the demand for environment-friendly, and sustainable aquaculture practices[2]. Probiotic supplements exert beneficial effects on the host by producing inhibitory compounds, competing for chemicals and adhesion sites, modulating and stimulating immune functions and improving the microbial balance and growth promoting factors[12].

Therefore, understanding the protection mechanisms in shrimp and their immune responses has become a priority among shrimp pathologists. Immune system of decapod crustaceans iscomposed of circulating haemocytes which are mainly semi-granular and granular cells involved in recognition of pathogens, prophenoloxidase (proPO) activating system and pathogen neutralizing actives such as encapsulation and coagulation[13]. Several types of haemocytes have the molecular mechanism for associating with a number of proteins in the prophenoloxidase system[14].

Most studies on the use of probiotics in aquaculture have focused on the use of single bacterial strain at one or more doses, either through the diet or through the culture environment[10]. The use of combination of two probiotic strains in the diet or the culture environment and their effects on growth, nutrient consumption and gut microbial composition is yet to be explored. As the strains were developed indigenously from the gut microbiota of marine prawn there will be no hindrance in the establishment and multiplication of probiotic bacterium in the digestive mileu of the animal. In this study the two probiotic microorganisms namely *Bacillus coagulans* and *Bacillus firmus,* individually and as combined bacterial diet were fed to *Penaeus monodon* so as to evaluate its growth parameters, biochemical constituents, Total Haemocyte Count (THC), prophenoloxidase activity, nitroblue tetrazolium activity (NBT) and the rate of *Vibrio* clearance in the haemolymph. The SEM analysis of the digestive tract was also done after bacterial supplementation and infecting with *Vibrio alginolyticus.*

MATERIALS AND METHODS

Experimental animal and design: The juvenile shrimps were weighed accurately in digital electronic balance before the start of the experiment. Post larvae (PL-20) of *Penaeus monodon* were collected from a private farm in Kancheepuram district, Tamil Nadu, India. Before acclimatization, they were washed for a few seconds in 0.1% benzalkonium hydrochloride to clear any adhering bacteria. Then the animals were washed three times in sterilized water and were acclimatized in 60 L PVC troughs for a week, under optimum hydrological conditions. The shrimps were fed daily at three intervals with formulated feed at 3% body weight.

The experimental animals were divided into 8 groups, comprising of 25 animals each. First group was kept as control group, fed only with normal formulated feed. Second group of animals were fed on normal formulated diet supplemented with *B. coagulans* (BSCB-2), third group was supplemented with *B. firmus* (BSCB-13), fourth group with combined bacterial diet (*B. coagulans+B. firmus*), fifth group fed with normal formulated feed (Infected), sixth group fed with feed supplemented with *B. coagulans* (BSCB-2), seventh group was fed on *B. firmus* (BSCB-13) and the eighth group was supplemented with combined bacterial diet (*B. coagulans+B. firmus*) group. The shrimps were kept on their respective diets for 14 days. On the 15 day the fifth, sixth, seventh and eighth groups were challenged with pathogenic *Vibrio alginolyticus* through injections while the rest of the groups were injected with appropriate quantities physiological saline. The feeding regimen continued till the 30th day when the experiment was culminated.

Table 1: Ingredients of prepared diets

Ingredients	Percentage
Fish meal	37.0
Prawn meal	15.0
Corn flour	0.5
Groundnut oilcake	20.0
Tapioca flour	20.0
Calcium carbonate	0.5
Calcium phosphate	3.0
Fish oil	5.0
Vitamin and mineral mix	2.5

Feed preparation

Compounded feed: Pelleted feed with essential nutrients in adequate composition, based on the recommendations of Tacon[15], was compounded in the laboratory. The composition of the feed given in Table 1.

All the components of the feed except mineral mix and fish oil were powdered finely in a kitchen mixer and autoclaved at 15 lbs, for 15 min. In lukewarm condition, fish oil and mineral mix were added to it and mixed thoroughly. The mixed feed was then extruded through a noodling device with holes of 3.5 mm diameter and dried in an oven set at 70°C. The dried feed-noodles were crushed and kept in airtight sterile containers.

Fatty acid methyl ester (FAME) analysis: Gas chromatographic analysis of whole cell Fatty Acid Methyl Esters (FAME) was performed for further identification and grouping of isolates. Fatty acid methyl ester extraction was performed using standard procedures by Sasser[16]. The fatty acid profiles generated were compared against an inbuilt Sherlock TSBA Library version 3.9 (MIDI Inc., DE, USA). A similarity index of >60% was used for clustering of isolates at species level.

DNA isolation and purification: Pure genomic DNA was isolated following the method of Ausubel *et al.*[17]. Briefly, the cultures were grown overnight in 3 mL nutrient broth with shaking at 30°C. A 1.5 mL quantity of the culture was centrifuged at 12,000×g for 10 min and the resultant pellet was resuspended in 567 µL 1X TE buffer (10 mM Tris pH 8.0, 1 mM EDTA). Proteinase K and SDS were added to final concentrations of 100 µg mL^{-1} and 0.5% respectively and incubated at 37°C for 1 h. After incubation, NaCl (5 M) and CTAB/NaCl (10% w/v cetyl trimethyl ammonium bromide in 0.7 M NaCl) were added and incubated at 65°C for 10 min. The mixture was extracted once, each with an equal volume of chloroform-isoamyl alcohol (24:1) and phenol-chloroform-isoamyl alcohol (25:24:1). DNA was precipitated from the aqueous phase using 0.6 volumes of isopropanol and washed once with 70% ethanol. The DNA pellet obtained after final centrifugation was vacuum dried and dissolved in 50 µL 1X TE buffer. The DNA quantification was done using a UV-1601 spectrophotometer (Shimadzu Corporation, Japan).

16S rDNA sequencing and AP-PCR: The 16S rDNA of two strains [*B. coagulans* (BSCB-2) and *B. firmus* (BSCB-13)] were PCR amplified using universal primers at PCR conditions described by Iwamoto *et al.*[18]. The resultant 454 bp products were purified using a PCR purification kit (Qiagen, Germany) and sequenced. The sequences were subjected to homology search using BLAST program of the National Center for Biotechnology Information (NCBI)[19]. The AP-PCR was performed using primer CRA22 described by Neilan[20]. All the reactions were carried out in 30 µL volumes consisting of 10X buffer (100 mM Tris-HCl, 500 mM KCl and 20 mM $MgCl_2$), 200 µM concentrations of each of the four dNTPs, 30 pmol of primer, 3 U of *Taq* polymerase (MBI Fermentas). All PCR amplifications were carried out in an Eppendorf mastercycler (Eppendorf, Germany). In all the reactions, 300 ng of the pure genomic DNA was used. The amplification products were separated on a 2% agarose gel, stained with ethidium bromide and photographed. Amplification profiles obtained were analyzed and a dendrogram was generated using BioNumerics version 4.6 software (Applied Maths, Belgium).

Feed coating: Putative probiotic strains of bacteria *B. firmus* and *B. coagulans* were isolated from the gut of wild marine *P. monodon.* Three groups of feed pellets were produced: 1) *B. coagulans* live cells (3.6×10^9 CFU g^{-1}), 2) *B. firmus* live cells (3.01×10^9 CFU g^{-1}) and 3) *B. coagulans+B. firmus* (3.9×10^9 CFU g^{-1}). Prepared feed pellets were warmed to 60°C and brought down to 35°C then blended with the molten agar containing respective bacteria. The mixture was stirred well with sterile glass rods to have a uniform coating of the bacteria over the feed pellets.

Pathogen challenge test: The LD_{50} was determined by employing probit analysis (Statistical package SPSS-10)[21]. After feeding the animals for two weeks with the bacteria supplemented feeds, prawns in each group were challenged with *V. alginolyticus* (MTCC 4182). All the shrimps were injected with 0.1 mL *V. alginolyticus* suspension in physiological saline intramuscularly at the LD_{50} dosage between their 3rd and 4th abdominal segments. The number of bacteria in the suspension was standardized by adjusting its absorbance in a spectrophotometer at 600 nm (1.0×10^3 CFU mL^{-1}) *V. alginolyticus* count was TCBS cultured

medium. The injected animals were observed for behavioural changes. Haemolymph and tissue were collected after treatment and biochemical parameters were analyzed.

Determination of growth parameters: Several growth parameters such as mean weight gain, mean length gain, Specific Growth Rate (SGR), Food Conversion Ratio (FCR), mean feed intake and survival were calculated for *Penaeus monodon* fed on formulated diets supplemented with the chosen putative probiotic bacterial species, either (1) *B. coagulans,* (2) *B. firmus*, or (3) Combined bacterial diet (*B. coagulans+B. firmus*). Feeding efficiency was calculated using the formula give by Kiessling and Askbrandt [22].

$$\text{Weight gain (\%)} = \frac{\text{Final weight (g)} - \text{Initial weight (g)}}{\text{Initial weight (g)}} \times 100 \quad (1)$$

$$\text{Length gain (\%)} = \frac{\text{Final length (cm)} - \text{Initial length (cm)}}{\text{Initial length (cm)}} \times 100 \quad (2)$$

$$\text{Specific Growth Rate (SGR) (\%)} = \frac{\ln w_2 - \ln w_1}{T_2 - T_1} \times 100 \quad (3)$$

where, ln is logarithm, W_1 is weight at time T_1 and W_2 is weight at time T_2.

$$\text{Feed Conversion Ratio (FCR)} = \frac{\text{Total dry feed intake (g)}}{\text{Wet weight gain}} \quad (4)$$

$$\text{Mean feed intake (g)} = \frac{\text{Total feed consumed (g)}}{\text{Initial number of animal} + \text{Final number of animal}/2} \times 100 \quad (5)$$

$$\text{Survival} = \frac{\text{Shrimp number at the end of experiment}}{\text{Shrimp number at the beginning of experiment}} \times 100 \quad (6)$$

Haemolymph collection: From random representative shrimps of each feed group before *Vibrio* infection and after infection, 200 µL haemolymph samples were withdrawn from the ventral sinus using a 1.0 mL syringe containing equal volume of sterile anticoagulant solution (30 mM trisodium citrate, 115 mM glucose, 10 mM EDTA, 26 mM citric acid, 338 mM NaCl, pH 4.6). Haemolymph samples collected from each group were mixed gently in Eppendorf tubes without centrifuging and used immediately.

Biochemical parameters: Biochemical constituents of PL were determined as total proteins[23], carbohydrates[24] and lipids[25]. Half the groups of shrimps were infected with *V. alginolyticus* on the 16th day and were sustained on the respective regimen of probiotic feeding along with uninfected shrimps. Biochemical parameters were analyzed after a total period of 30 days for the infected and the non infected shrimps.

Immune parameters

Total Haemocyte Count (THC): Total haemocyte count was made using an improved Neubauer haemocytometer following the method as described for counting WBC[26].

Prophenoloxidase activity: Phenoloxidase activity was measured spectrophotometrically by recording the formation of dopachrome produced from L-dihydroxy phenylalanine (L-DOPA, Hi Media, Mumbai) following the method described by Felix and Sivakumar[27].

Nitroblue tetrazolium activity (NBT): Nitroblue tetrazolium was determined by the method of Song and Hsieh[28].

Scanning Electron Microscope (Vega III Tescan-USA) analysis of the intestine of shrimps supplemented with probiotics: After probiotic supplementation for 15 days, shrimps were randomly selected and kept on ice. Their gastrointestinal tracts were dissected out. The intestine were longitudinally cut and rinsed vigorously with sterile saline three times before fixation and a portion was fixed in 2.5% (w/v) Glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) for 2 h. After fixation these samples were processed by conventional procedure as reported previously by Watson *et al.*[29]. Scanning Electron Microscopy (SEM) was done using a VEGA III TESCAN-USA electron microscope.

Bacterial clearance test: Shrimps were acclimatized in laboratory conditions for a period of 15 days. A bacterial suspension (*V. alginolyticus*) of 0.1 mL (1.0×10^3 CFU mL^{-1}) was injected into the tail muscle of each shrimp. Then they were kept in seawater aquaria equipped with aeration for three hours. Haemolymph was collected from each shrimp without anticoagulant and 30 µL of haemolymph was dropped on thiosulfate-citrate-bilesalt-sucrose (TCBS) agar (Himedia, Bangalore). A two-fold dilution of the whole haemolymph was made using sterile 2.6% NaCl solution. Haemolymph was withdrawn every three hours and plated for *Vibrio* in TCBS medium. The colonies were enumerated after 48 h incubation.

Statistical analysis: All the values were expressed as Mean±Standard Deviation (SD). The statistical significance was evaluated by two-way Analysis of Variance (ANOVA) using

Table 2: Growth parameters of *Penaeus monodon*, supplemented with live probiotics *B. coagulans*, *B. firmus* and combined bacterial feed (Mean values±Standard Deviation)

Growth parameters	Control	*B. coagulans*	*B. firmus*	Combined bacterial diet
Initial weight (g)	0.333±0.001^{d}	0.354±0.001^{c}	0.528±0.001^{b}	0.465±0.001^{a}
Final weight (g)	0.949±0.001^{d}	1.295±0.001^{c}	1.624±0.001^{a}	1.577±0.001^{b}
Initial length (cm)	3.54±0.01^{d}	3.61±0.01^{c}	4.21±0.01^{a}	3.94±0.01^{b}
Final length (cm)	5.16±0.01^{d}	5.84±0.01^{c}	6.32±0.01^{b}	6.30±0.01^{b}
Mean weight gain (g)	0.61±0.01^{d}	0.94±0.01^{c}	1.09±0.01^{b}	1.11±0.01^{a}
Weight gain (%)	185.69±0.8^{d}	265.10±1^{a}	207.89±1^{c}	239.18±1^{b}
Mean length gain (cm)	1.62±0.01^{d}	2.23±0.01^{b}	2.11±0.01^{c}	2.36±0.01^{a}
Length gain (%)	46.91±1^{c}	62.79±1^{a}	51.11±1^{b}	61.04±1^{a}
Food Conversion Ratio (FCR)	3.24±0.01^{a}	3.11±0.03^{b}	1.80±0.01^{d}	2.89±0.02^{c}
Specific Growth Rate (SGR)	4.13±0.01^{d}	6.29±0.02^{c}	7.32±0.02^{b}	7.41±0.01^{a}
Mean feed intake (g)	1.94±0.03^{a}	1.58±0.5^{a}	2.07±0.02^{a}	2.03±0.05^{a}
Survival (%)	21.6±1^{d}	68.6±1^{b}	76±2^{a}	42±2^{c}

ANOVA ($p<0.05$), SNK test: Dissimilar superscripts denote statistically significant different values

SPSS version 10.5 (SPSS, Cary, NC, USA) and the individual comparisons were obtained by Post-hoc analysis, 'Student Newman Keuls' test (SNK)[30].

RESULTS

In the present study, marine shrimp *Penaeus monodon* were fed with two putative probiotics individually and in combination to evaluate growth parameters such as mean weight gain, mean length gain, FCR, SGR and mean feed intake and the results are summarized in Table 2. Biochemical parameters and immune parameters were analyzed to substantiate the results (Table 3, 4). Further, gut colonization by bacteria was analyzed using SEM observation of intestine histological sections.

Taxonomic identification of *Bacillus* spp. isolated from gut of *P. monodon*: *Bacillus coagulans* and *B. firmus* were isolated and identified by biochemical tests and Fatty Acid Methyl Ester Analysis (FAME). For the Bacillus species, GC group 22 corresponds to the gas chromatographic profile of a *Bacillus* species in the Sherlock TSBA Library version 3.9 (Microbial ID, MIDI Inc.), the 16S rDNA sequence of which match with known species of the genus *Bacillus.*

Growth and survival rate after feeding and infectivity trial: After the feeding trial, it was found that the mean weight gain (%) increased significantly ($p<0.05$) in all the three probiotic supplemented groups compared to the control (185%) with the highest value in *B. coagulans* (265%) fed group followed by combined bacterial diet (239%) and *B. firmus* (207%) fed groups. Similar results were obtained for mean length gain. Maximum FCR was observed in *B. coagulans* (3.11) supplemented group. Highest mean feed intake (2.07) and survival rate (76 %) were observed in *B. firmus* supplemented group.

Biochemical composition after probiotic supplementation and *V. alginolyticus* infection: The biochemical composition of shrimps fed with experimental diet are presented in Table 3. The total proteins, carbohydrates and lipids of the shrimp showed significant difference ($p<0.05$) between the control and the experimental groups. In muscle tissues, the protein content (87.9 mg g^{-1}) was highest in the combined bacterial diet fed groups, where as carbohydrates (14.3 mg g^{-1}) and lipids (0.33 mg g^{-1}) were highest in *B. firmus* supplemented group. However, after infection with *V. alginolyticus*, muscle tissues of combined bacteria fed shrimps recorded highest levels in all biochemical components. Similar results were obtained for the biochemical components of hepatopancreas after probiotic supplementation of the shrimps. After infection, shrimp hepatopancreas recorded maximum protein (98.9 mg g^{-1}) content in the *B. coagulans* supplemented group and for lipids (0.33 mg g^{-1}) and carbohydrates (16.5 mg g^{-1}), *B. firmus* supplemented group recorded the highest values.

Immune response: Before *V. alginolyticus* infection of the shrimps, the higher Total Haemocyte Count (THC), prophenol oxidase activity and NBT activity were observed in all the probiotic supplemented groups compared to the control ($p<0.05$) (Table 4). The THC (1584) and NBT (4.21) were observed in *B. firmus* supplemented group, however higher prophenol oxidase activity was observed in *B. coagulans* (7.3 U min^{-1} mg^{-1} of protein) supplemented group.

After infection with *V. alginolyticus,* a significant difference was observed between the control and the probiotic supplemented groups ($p<0.05$). *Bacillus firmus* group recorded higher THC with 1049, while higher prophenol oxidase and NBT activity were observed in *B. coagulans* fed shrimps.

Bacterial clearance: Clearance of *Vibrio* cells from the shrimp haemolymph at 5 min, 10 min, 2 h and 12 h of

Table 3: Biochemical components of normal and *V. alginolyticus* challenged *Penaeus monodon*, supplemented with live probiotics *B. coagulans*, *B. firmus* and combined bacterial diets (Mean values±Standard Deviation)

Experiments	After supplementation				After supplementation and infection			
Diets	Control normal	*B. coagulans*	*B. firmus*	Combined bacterial diet	Control infected	*B. coagulans*	*B. firmus*	Combined bacterial diet
Muscle								
Protein (mg g^{-1})	63.5±0.1^{d}	73.2±0.01^{c}	70.9±0.01^{b}	87.9±0.01^{a}	70.9±0.01^{d}	82.2±0.01^{c}	87.0±0.01^{b}	89.0±0.01^{a}
Carbohydrate (mg g^{-1})	7.2±0.1^{d}	8.3±0.1^{c}	14.3±0.3^{b}	11.8±0.3^{a}	8.6±1.0^{d}	10.3±0.2^{c}	19.8±0.1^{b}	34.4±0.4^{a}
Lipid (mg g^{-1})	1.7±0.1^{d}	2.1±1.0^{c}	3.3±0.3^{b}	2.5±0.3^{a}	1.5±1.0^{d}	1.7±0.2^{c}	2.3±0.1^{b}	3.9±0.4^{a}
Hepatopancreas								
Protein (mg g^{-1})	40.6±0.1^{d}	53.6±0.1^{a}	47.9±0.2^{c}	67.0±0.2^{b}	73.6±0.2^{d}	98.9±0.3^{a}	90.6±0.1^{c}	80.6±0.3^{b}
Carbohydrate (mg g^{-1})	9.7±0.1^{d}	10.2±0.1^{c}	19.3±0.2^{b}	29.1±0.1^{a}	9.3±0.1^{d}	9.6±0.2^{c}	16.5±0.1^{b}	11.6±0.2^{a}
Lipid (mg g^{-1})	0.22±0.1^{c}	0.38±1.0^{a}	0.29±0.3^{b}	0.32±0.3^{b}	0.24±1.0^{c}	0.25±0.2^{a}	0.33±0.1^{b}	0.29±0.4^{b}

ANOVA ($p<0.05$), SNK test: Dissimilar superscripts denote statistically significant different values

Table 4: Immune parameters of normal and *V. alginolyticus* challenged *Penaeus monodon*, supplemented with live probiotics *B. coagulans*, *B. firmus* and combined bacterial diets (Mean values±Standard Deviation)

Experiments	After supplementation				After supplementation and infection			
Diets	Control normal	*B. coagulans*	*B. firmus*	Combined bacterial diet	Control infected	*B. coagulans*	*B. firmus*	Combined bacterial diet
THC	961±16^{d}	1477±60^{c}	1584±6^{a}	1406± 4^{b}	561±5.0^{d}	731±8^{c}	1049±10^{a}	921±3^{b}
ProPO (U min^{-1} mg^{-1} of protein)	1.8±0.1^{c}	7.3±0.2^{a}	3.8±0.3b	4.1±0.1^{b}	1.7±0.1^{c}	2.8±0.2^{a}	2.3±0.1^{b}	2.5±0.2^{b}
NBT*	0.25±0.1^{d}	3.83±0.3^{b}	4.21±0.2^{a}	1.77±0.1^{c}	0.88±0.1^{d}	1.74±0.3^{b}	1.69±0.2^{a}	1.41±0.1^{c}

ANOVA ($p<0.05$), SNK test: Dissimilar superscripts denote statistically significant different values, *Relative production of O_2 = OD_{630} of stimulated haemocytes/OD_{630} of control haemocytes

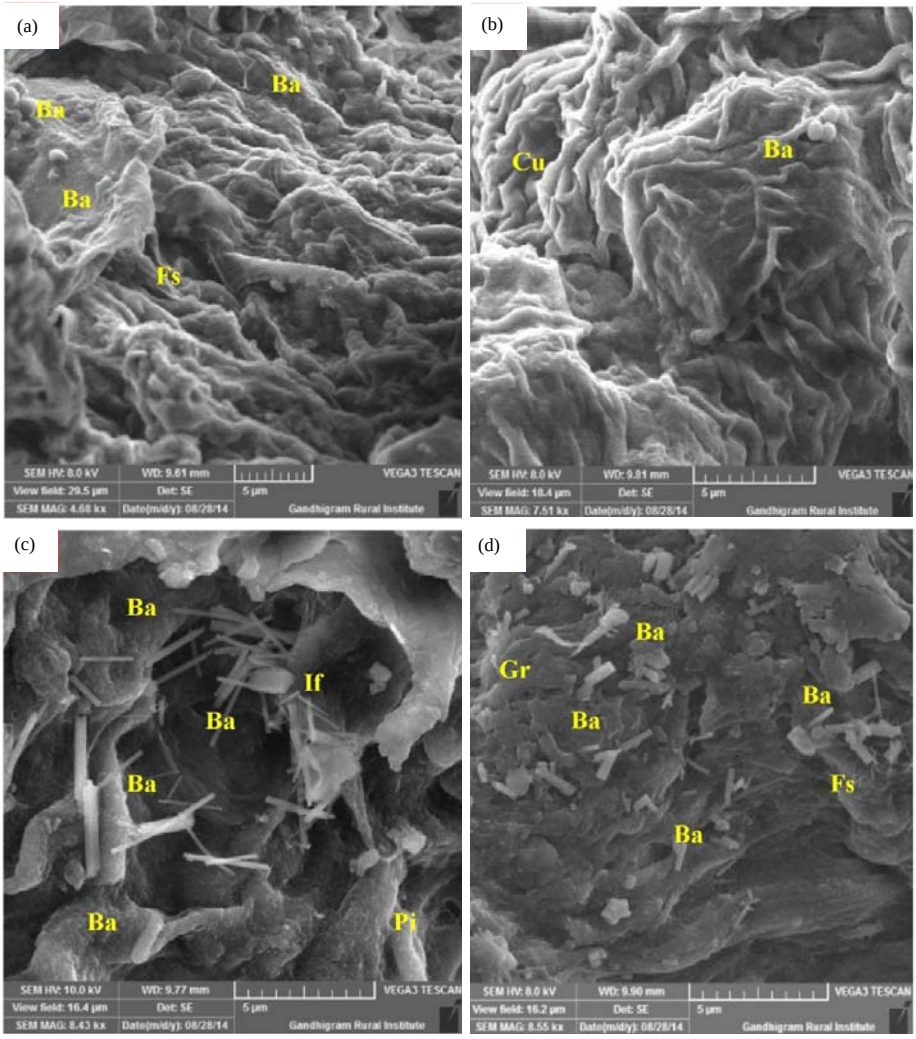

Fig. 1(a-d): Representative scanning electron microscopy micrographs of the inner surface of the digestive tract of farmed *Penaeus monodon*. (a) Control, (b) *B. coagulans* fed, (c) *B. firmus* fed and (d) Combined bacterial diet fed
Ba: Bacteria, Gr: Granule , If: Ingested food, Pi: Pit, Fs: Fiber seta, Cu: Cuticle

infection was studied by calculating the reduction in bacterial colonies by plating periodic samples of haemolymph on TCBS agar. There was an initial spurt (5 min after injection) in bacterial number of the haemolymph due to *Vibrio* injection and this spurt was lesser in the probiotic maintained shrimps (Table 5). After 12 h, the number of vibrios came down to three fourth the level of the initial spurt in normal shrimps. In the two probiotic supplemented shrimps, the reductions in vibrios varied between 60-66% of the initial numbers after 12 h infection. Although the clearance rate was comparatively lesser in the probiotic supplemented shrimps by virtue of the lower degree of spurt initially, the net numbers of *Vibrio* remained substantially lower in the haemolymph of probiotic supplemented shrimps after 12 h of clearance. Between the two probiotic bacteria, *B. firmus* had a greater influence on *Vibrio* clearance. Although probiotic supplementation induced the proliferation of haemocytes their efficiency in phagocytosis was not apparent in the context of bacterial clearance.

Scanning electron microscopic analysis of shrimps-gut: Bacterial cells were found singly scattered on the plasma membrane of the gastrointestinal tract (Fig. 1a, b, d). Clusters of granules inside the cytoplasm of the epithelial cells were seen protruding through the microvilli into the lumen of gut. A few bacterial cells were seen in the lumen of the gut, these were principally short-rod shaped bacteria attaching to the inner surface or in small pits scattered on the inner surface of the gut (Fig. 1c, d).

Table 5: *Vibrio alginolyticus* clearance in *Penaeus monodon*, supplemented with live probiotics *B. coagulans, B. firmus* and combined bacterial diets (Mean values±Standard Deviation)

Time	Control (CFU mL^{-1})	*B. coagulans* (CFU mL^{-1})	*B. firmus* (CFU mL^{-1})	Combined bacterial diet (CFU mL^{-1})
5 min	218.3±7^{a}	171.6±10^{b}	132.0±4^{d}	156.3±5^{c}
10 min	176.3±7^{a}	107.3±2^{b}	139.0±3^{d}	135.0±5^{c}
2 h	165.0±5^{a}	137.6±2^{b}	103.0±6^{d}	110±2^{c}
12 h	171.6±7^{a}	112.6±3^{b}	84.0±5^{d}	97.6±7^{c}

ANOVA ($p<0.05$), SNK test: Dissimilar superscripts denote statistically significant different values

DISCUSSION

Administering probiotics in live form in aquaculture is currently on the increase. It is considered a healthy approach to minimize health related problems in the host animal, to enhance their immunity and to reduce the pathogen menace in aquaculture. In the present study, we determined the growth performance, survival, biochemical constituents and immune expression of shrimps, *P. monodon* fed with diets containing of probiotic bacteria namely *B. coagulans* and *B. firmus* separately in and in combined form upon infection with *V. alginolyticus*, further in infected shrimps probiotic supplementation increased the mean weight gain, mean length gain, SGR, FCR, mean feed intake and survival rate and they were all on par with similar parameters observed in uninfected animals (Table 2). These findings are in agreement with the previous reports in shrimp[31-33]. Combined probiotic bacterial supplementation to the shrimp culture for the improvement of SGR, FCR, survival and immune response of shrimp was reported by Van Hai and Fotedar[34]. In *L. vannamei,* Rainbow trout [35], Nile tilapia[36], *Aequidens rivulatus*[37] and in *Labeo rohita*[38] also similar observation could be made.

Gatesoupe[2] suggested that probiotics may improve digestion through exoenzyme secretion. Enhanced growth in shrimp inoculated with *Bacillus* spp. was demonstrated by Gullian *et al.*[39] and El-Dakar and Goher[40]. *Bacillus* probiotics are capable of secreting lipase, a key enzyme, which triggers of essential fatty acids for enhanced growth and confer immunity to shrimps[41,42]. The data obtained in the present study indicate that irrespective of species or combination, the probiotics promote increased growth and immune expression. Probiotic supplemented shrimps yielded higher protein, carbohydrates and lipids compared to the control group. These results also agree with the pattern of biochemical changes in *M. rosenbergii* fed with *L. sporogenes* [33].

In crustaceans, circulating haemocytes play an important role in the immune response and the circulating haemocytes level can vary with the duration of infection[43,44]. The THC of *P. monodon* sustained on probiotic supplemented diet was much higher than that of the control shrimps. Similar observations were reported in *M. rosenbergii*[45], *P. latisulcatus*[34], *L. vannamei*[46] when supplemented with probiotic strains. Substantial reduction in THC of *P. monodon* was observed presently when challenged with *V. alginolyticus* and this was in accordance with the results observed by De La Pena *et al.*[47] in *P. monodon* and *Marsupenaeus japonicus* infected with WSSV and in shrimps affected by Taura syndrome[48].

The prophenol Oxidase system is considered the main immune system in crustaceans[49]. The ProPO system activation is involved with the release of some important molecules to perform crucial immune responses including non-self recognition, melanin formation, adhesion and cell-cell communication[50]. Prophenol oxidase activity in haemolymph was higher in the shrimps fed with probiotic supplemented diets. Studies have indicated increased immune responses with probiotic supplementation in *L. vannamei*[51] and *P. japonicus*[52]. Rengpipat *et al.*[53] reported increased prophenol oxidase activity in *P. monodon* on supplementation with *Bacillus* species. It was revealed in the earlier studies that a combination of probiotic bacterial strains complement each other and occupy different niches with in the gut microfloral environment and thus could result in the enhancement or prolongation of desirable effects on host immune response and health[54]. The present study also confirms the above hypothesis.

Nitroblue tetrazolium reduction assay is widely used to detect the production of superoxide anion to quantify Respiratory Burst (RB) activity of phagocytes cells. Previous studies have suggested respiratory burst activity with increased resistance to various pathogens in different shrimp species[55]. The results of the present studies clearly indicate that addition of probiotic increases the respiratory burst activity and this corroborates the findings of Liu *et al.*[56] in *L. vannamei* and Zhang *et al.*[52] in *P. japonicus.*

Pathogen clearance efficiency in the shrimps was assessed as another disease combating mechanism. In crustaceans, clearing of viable cells in haemolymph was observed by Adams[57]. The number of bacteria, after 8-12 h in Kurma shrimp *M. japonicus* when injected with *Vibrio* and the number of live bacteria decreased to 50 and 3% after 10 min and 2 h respectively in the tiger shrimp *P. monodon* injected with *V. anguillarum*[58]. Increased clearance efficiency was absent in probiotic *L. rhamnosus*, supplemented and

V. alginolyticus challenged *L. vannamei*[51]. In the present study also live bacteria in the haemolymph decreased when injected with *V. alginolyticus. Vibrio* clearance efficiency was particularly pronounced in shrimps maintained on *B. firmus* diet. The SEM analysis could reveal the colonization of intestinal crypts by viable bacterial strains, thus revealing the sites of action by the probiotic bacterial strains.

CONCLUSION

It is concluded from the present study that live probiotic microorganisms may be incorporated while formulating shrimp diet this imparts beneficial effects on the growth of *Penaeus monodon* and can remarkably improve disease resistance by modulating intestinal microflora thereby stimulating the immunity in shrimps.

SIGNIFICANCE STATEMENT

Bacterial strains currently incorporated in commercial probiotic are intended for terrestrial live stock and not specific for marine shrimp and their perceived disease fighting action seems to the incidental. Two putative probiotic marine bacterial strains *B. coagulans* (BSCB-2) and *B. firmus* (BSCB-13), autochthonous in nature and specific for the shrimps *Penaeus monodon* were tested for their growth promoting and immune enhancing properties through feeding experiments. The positive results obtained at present will help the shrimp farmers to understand the scientific basis of probiotic action and also give a guideline for selecting specific probiotics.

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