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Original Article

Survival and Growth Promoting Effects of *Bacillus Subtilis* on *Macrobrachium Rosenbergii*, and Validation of Gut Microflora

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ABSTRACT

This study emphasized the survival and growth of the commercially important freshwater prawn, *Macrobrachium rosenbergii* post-larvae on formulated diets supplemented with a probiotic bacterium, *Bacillus subtilis* at five different serially diluted concentrations (10^{-1}, 10^{-3}, 10^{-5}, 10^{-7} and 10^{-9}). After 90 days of feeding, 10^{-7} (CFU, 2.76×10^{-7}) concentration produced the best results in increased survival rate, weight gain, basic biochemical constituents (total protein, amino acid, carbohydrate and lipid) and activities of digestive enzymes (protease, amylase and lipase) when compared with control diet prepared without *B. subtilis*. This was due to maintenance of good intestinal health, because of *B. subtilis* supplementation. The presence of *B. subtilis*, *Bacillus cereus*, *Lactobacillus delbrueckii* (subsp. *bulgaricus*), *Lactobacillus casei*, *Lactobacillus rhamnosus* and *Streptococcus pyogenes* have been identified in the gut of *M. rosenbergii* fed with *B. subtilis* supplemented diet, through 16S r-RNA gene sequencing which was authenticated with NCBI GenBank. The pathogenic bacteria such as *Escherichia coli*, *Klebsiella* spp., and *Staphylococcus* spp., present in the control prawns as per our previous study (Manjula *et al*., 2018), were competitively excluded by the experimental prawns fed with *B. subtilis* supplemented diet in the present study. Therefore, *B. subtilis* is recommended for sustainable production of *M. rosenbergii* seed.

Key words: *M. rosenbergii*, *B. subtilis* growth, Protein, 16S r-RNA gene

Introduction

In the natural culture system, the required nutrients may come from water itself through many biotas for any animal. In artificial culture system, it should majorly be supplied from prepared diets, which must be nutritionally complete with all essential nutrients. The freshwater prawn, *Macrobrachium rosenbergii* is fast growing in nature, domesticated and earns valuable foreign exchange. Therefore, this species is recommended for farming in many tropical and sub-tropical countries (Brown, 1991).

Feed formulation is a major input in the hatchery and the availability of cost-effective feeds play an important role in aquaculture industry (Chunchom *et al*., 2010). Prawn requires high protein diet for faster growth under intensive culture operation. Commercially formulated feeds contain 30 to 45% crude protein (Mitra *et al.,* 2005). Consequently, high protein feed stuffs, such as squid meal, fishmeal, prawn meal, clam meal, animal by products and oil seeds make up to 60% or more of the ingredients in commercial prawn feed. The other feed stuffs suitable for prawn feed formulation are grains, oil cakes (coconut, groundnut, linseed, safflower, sunflower, soybean *etc.*,), root/tubers of sweet potato and tapioca, cereals (rice, sorghum, wheat, maize *etc.*,), pulses (black gram, green gram, red gram, horse gram, cow pea *etc.*,), leaf protein concentrate, cane molasses, brewer's Yeast, *etc*.

Selection of ingredients for formulation of feed can be made based on local availability and cost, particularly for protein sources. The protein intake is critical for normal growth, survival, and body functions as it has the building blocks, the essential amino acids, such as methionine, valine, threonine, lysine, arginine, histidine, isoleucine, leucine, phynylalanine and tryptophan (Millamena *et al*., 1999). Under laboratory conditions, the protein requirement of post larvae and juveniles are 30-45% and the optimum P/E ratio is reported to be 26.28 mg protein/KJ digestible energy (Balazs and Ross, 1976; Balaji *et al*., 2002). According to (New *et al.*, 2010), protein level of 25% or less, produce acceptable growth for *M. rosenbergii* culture in earthen ponds. The juveniles are able to digest lipids efficiently. Its apparent assimilation efficiency is 94-97% (Newman *et al.,* 1982). In *M. rosenbergii*, the highest weight gain has been reported when fed with diets containing a mixture of 18:3 n-3 and 18:2 n-6 (Teshima *et al.,* 1992). The protein sparing on growth will be enhanced, if adequate quantum of carbohydrate is provided.

The probiotic bacteria are live microorganisms which when administrated in adequate amounts confer a health benefit on the host. Supplementations of probiotic bacteria in appropriate concentration derive health-promoting benefits (Sharma *et al*., 2012). The potential benefits of probiotics in aquaculture include improvement of water quality and enhancement of nutrition of the host through production of vitamins, minerals, trace elements and digestive enzymes, led to lower incidence of diseases due to improved immune response, which in turn ultimately produced greater survival and growth (Bidhan *et al*., 2014; Ghosh *et al*., 2004, 2008; Holzapfel *et al.,* 1998, 2001). Although probiotics for human and terrestrial animals are dominantly lactic acid bacteria, many different genera, including Yeast, *Saccharomyces cerevisiae, Bacillus* spp., and *Lactobacillus* spp., has been evaluated as probiotics on prawn and fish (Kavitha *et al.,* 2018; Seenivasan *et al*., 2011, 2012, 2013, 2014).

Probiotics actively inhibit the colonization of potential pathogens in the digestive tract by antibiosis or by competition for nutrients and space, as well as alteration of the microbial metabolism, stimulating host immunity and disease resistance, which differs based on the defense mechanism of the host (Aly, 2009; Cruz *et al*., 2012; El-Haroun *et al.,* 2006; Hoseinifar *et al*., 2018). *Bacillus* spp., *Lactobacillus* spp., and *Enterococcus* spp., are used widely as probiotics in freshwater prawn for improvement of survival, growth and better feed conversion (Jain *et al*., 2020a, b; Jayanthi *et al*., 2015a, b; Karthik and Bhavan, 2018; Karthik *et al*., 2018a, b; Manjula *et al*., 2018; Narmatha *et al*., 2017; Sudha *et al*., 2019).

Bacillus subtilis is a Gram-positive lactic acid-forming bacterium. A new strain of *B. subtilis* BI19, which produces extracellular amylase, has been identified and characterized through 16S r-DNA (Dash *et al*., 2015). It has been reported that *B. subtilis* 2335 produce the antibiotic, Amicoumacin with *in-vitro* activity against *Helicobacter pylori* (Pinchuk *et al*., 2001; Urdaci and Pinchuk, 2004). A serine protease, Nattokinase secreted from vegetative cells of *B. subtilis* reduces blood clotting by fibrinolysis (Agrebi *et al*., 2009; Wang *et al*., 2011; Mukherjee *et al*., 2012; Fadul *et al*., 2015). It has been reported that *B. subtilis* and *Bacillus licheniformis* (BioPlus2B) improve resistance to infection by *Yersinia ruckeri* (Raida *et al.,* 2003) in the trout; a probiotic mixture of *B. subtilis* and *Bacillus cereus* provides disease resistance due to improved non-specific immunity in sea cucumber, *Apostichopus japonicas* (Li *et al*., 2015); *B. subtilis* controls *Aeromonas hydrophila* infection in Indian major carp, *Labeo rohita* (Kumar *et al*., 2006); *B. subtilis* BT23 and *Bacillus* S11 control *Vibrio harveyi* infections in black tiger shrimp, *P. monodon* (Rengpipat *et al*., 2003; Vaseeharan and Ramasamy, 2003); *B. subtilis* administration to channel catfish, *Ictalurus punctatus* and striped catfish, *Pangasianodon hypophthalmus* reduce mortalities produced due to *Edwardsiella ictaluri* infection (Ran *et al*., 2012).

The present study aimed at supplementing *B. subtilis* through formulated diet to *M. rosenbergii* PL. The parameters studied include nutritional indices (survival rate, weight gain and food conversion), activities of digestive enzymes (protease, amylase and lipase), contents of basic biochemical constituents (total protein, total amino acid, total carbohydrate and total lipid) and 16S r-RNA gene sequencing of bacterial consortium established in the gut of *M. rosenbergii* PL fed with *B. subtilis* supplemented diet.

Experimental

Procurement of *B. subtilis* (MTCC 121) and its sub-culture

Pure culture of the probiotic bacterium, *B. subtilis* was procured from Microbial Type Culture Collection (MTCC 121), Chandigarh, India, in lyophilized powder form. The nutrient culture medium containing peptic digest of animal tissue (5.0 g/L), sodium chloride (5.0 g/L), Beef extract (1.5 g/L) and *Yeast* extract (1.5 g/L) was prepared. The medium (1.3 g) was mixed with distilled water (100 mL) and autoclaved at 121 °C for 15 minutes (pH, 7.4 at 25 °C). The broth was later dispensed into 100 mL sterile conical flask. A loop-full of lyophilized powder of *B. subtilis* was inoculated into the broth. The culture flask was placed on a shaken incubator for 12 hours at 37 °C. The clear broth turned into turbid, which indicates the growth of *B. subtilis* (Figure 1). The bacterial cells were harvested by centrifuging at 5000 rpm for 10 minutes. Actually, the supernatant was filtered by passing through a 0.25 µM silica gel coated membrane (Himedia, India), washed twice with phosphate-buffered saline (pH 7.2), weighed and re-suspended in the same buffer. It was stored at 4 °C, and used for further study.

Agar (12.0 g/L) was added to the nutrient culture medium and 30 µl of *B. subtilis* suspension was spread over the agar medium and incubated for 12 hours at 37 °C. The appearance of white colonies was observed (Figure 2). In order to optimise the concentration of *B. subtilis*, the culture broth was serially diluted up to 10^{-9} and seeded on nutrient agar to see the CFU in each dilution and colonies observed were 5.87×10^{-1}, 4.72×10^{-3}, 3.13×10^{-5}, 2.76×10^{-7}, and 1.11×10^{-9}.

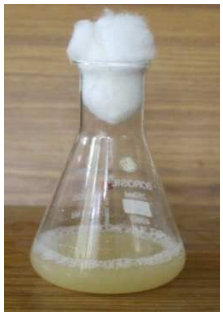

Figure 1. Culture morphology of *Bacillus subtilis* in nutrient broth

Figure 2. Culture morphology of *Bacillus subtilis* on nutrient agar spread plate

Feed preparation

The micro pulverized and sieved basal ingredients, such as fishmeal (25%), groundnut oil cake (25%), soybean meal (25%) were used as protein sources, and wheat bran (10%) was used as carbohydrate source. These ingredients were taken at different ratio based on Pearson's square method to maintain 40% protein level, thoroughly mixed and steam cooked for 15 min at 95-100 °C. After cooling at room temperature vitamin *B*-complex with vitamin C (1%) was added. Then tapioca flour (5%) and egg albumin (7%), and sunflower oil (2%) were added as binding agents and lipid source, respectively. The proximate composition and mineral contents of the control feed prepared were as follows (Manjula *et al*., 2018): crude protein, 45.88%; total nitrogen-free extract, 33.55%; crude fat, 7.28%; ash, 7.25%; moisture, 11.71%; gross energy, 4395 kcal/kg; sand and silica (acid insoluble ash), 0.88%; calcium, 0.90%; phosphorus, 0.82%; iron, 186.87 ppm; copper, 34.86 ppm; salt, 0.56% (AOAC, 2005; APHA, 2005). *B. subtilis* was supplemented with the basal diet at different serially diluted concentrations with different colony forming units (CFU), such as 5.87×10^{-1}, 4.72×10^{-3}, 3.13×10^{-5}, 2.76×10^{-7} and 1.11×10^{-9}. The dough was prepared with 10% boiled water, manually pelletized into 3.0 mm diameter and dried under room temperature until the moisture content reached below 10%. In order to maintain the probiotic viability, the pelletized fed with smooth surface was prepared once in 15 days.

Procurement and acclimatization of *M. rosenbergii* PL

The post larvae (PL-18) of the freshwater prawn, *M. rosenbergii,* were procured from a prawn culture nursery pond, Singanallur, Coimbatore, India. They were transported to the laboratory in polythene bags filled with oxygenated water. The prawns were acclimatized to the ambient laboratory condition with ground water for two weeks in cement tanks. The diluents control (ground) water had these physicochemical parameters: Temperature (°C),

23±0.2; pH, 7.2±0.20; total dissolved solids (TDS (g/L), 0.97±0.07; Dissolved oxygen (DO_2 (mg/L), 4.10±0.30; Salinity (mg/L), 0.64±0.01; Electrical conductivity (EC (Ms/cm), 1.02±0.01; Ammonia (mg/L), 0.031±0.007. During acclimatization the prawns were fed with boiled egg albumin and artificially prepared feed of our own laboratory. In order to maintain a healthy environment, >50% of tank water was renewed daily and adequately aerated. This ensures sufficient oxygen supply to the prawn and an environment devoid of accumulated metabolic wastes. The unfed feed, faeces, moulted exuvia and dead prawns, if removed by siphoning without disturbing the live prawns.

Feeding trail

M. rosenbergii (≠ PL-35) with 2.86±0.05 cm length and 0.39±0.05 g weight were used in this experiment. The prawns were divided into six experimental groups each consisted of 40 PLs housed in 35 L ground water. The experiment was conducted in triplicates for 90 days. Five experimental groups were fed with serially diluted concentration of *B. subtilis* incorporated diets such as 5.87×10^{-1}, 4.72×10^{-3}, 3.13×10^{-5}, 2.76×10^{-7} and 1.11×10^{-9} CFU, respectively. The control group was fed with basal diet formulated without incorporation of *B. subtilis* while renewing the water medium, the unfed feed, moulted exuvia and dead prawns if any were siphoned out daily without severe disturbance to the live prawns. On the 90th day of experiment the survival rate was calculated and the morphometric data, such as the final length and weight were measured for evaluation of nutritional indices, assays of digestive enzymes activities and estimation of concentrations of basic biochemical constituents.

Calculation of nutritional indices

Nutritional indices, such as survival rate (SR), weight gain (WG) and food conversion ratio (FCR) were calculated by adopting following equations (Tekinay and Davies, 2001).
Survival rate (%) = Total No. of live animals/Total No. of initial animals × 100
Weight gain (g) = Final weight (g) – Initial weight (g)
Food conversion ratio (g) = Total Feed intake (g)/Total weight gain of the prawn (g)

Assays of digestive enzymes

Activities of digestive enzymes were assayed on 60th day of feeding trial. The digestive tract of three prawns from each replicate group were carefully dissected out, homogenized in ice-cold distilled water and centrifuged at 9000 g under 4 ºC for 20 min. The supernatant was used as crude enzyme source. Total protease activity was determined by casein-hydrolysis method of (Furne *et al*., 2005), where one unit of enzyme activity represents the amount of enzyme required to liberate 1 µg of tyrosine per minute. Amylase activity was determined by (Bernfeld *et al*., 1955) method, the specific activity of amylase was calculated as milligrams of maltose liberate per gram of protein per hour (mg/g/h). Lipase activity was assayed by the method of (Furne *et al*., 2005), one unit of lipase activity was defined as the amount of free fatty acid release from triacylglycerol per unit time.

Estimations of basic biochemical constituents

Concentrations of total protein, amino acid and carbohydrate were estimated by adopting (Lowry *et al*., 1951; Moore and Stein, 1948; Roe, 1955) methods, respectively. The total lipid

was extracted by (Folch *et al*., 1957) method, and estimated gravimetrically by (Barnes and Blackstock, 1973) method.

Data analysis

Data between control versus experiments and between experiments were subjected to statistical analysis through one-way ANOVA and subsequent post hoc multiple comparison with DMRT by adopting SPSS (v20). All the details of statistical analyses were given in tables. The *P*-values less than 0.05 were considered as statistically (95%) significant.

Analysis of gut microbial consortium

The gut of both control, and experimental prawns fed with the best concentration of *B. subtilis* (CFU=2.76×10^{-7}) were subjected to bacterial culture. The prawns were deactivated by keeping them in freezer at -20 ºC for 10 minutes. Then, the surface was sterilized with 50 ppm formalin for 30 seconds, in order to remove the external microflora if any. Then, the digestive tract was dissected out individually and homogenized with phosphate buffered saline (pH, 7.2) under aseptic condition. Afterwards, the homogenates were serially diluted up to 10^{-4}. From each homogenate, 0.5 mL of aliquot was mixed with nutrient broth and kept for 24 h at 35 ºC. Then, 0.1 mL of broth culture was seeded over the surface of freshly prepared nutrient agar plate and incubates at 37 ºC for 24 h. Different bacterial colonies were identified and confirmed through routine bacteriological tests (Holt *et al*., 1996). The bacterial colony was enumerated using the formula, Bacteria count (CFU/g) = Number of colonies × Dilution factor/Volume of sample (g).

Molecular analyses

Isolation and purification of genomic DNA

Bacterial genomic DNA was isolated from individual culture, *Bacillus* spp., (two colonies) *Lactobacillus* spp., (three colonies) and *Streptococcus* sp., (one colony) using phenol, chloroform, iso-amyl alcohol (PCI) method. They were homogenized with 2 volume of cold TE buffer (500 µl). 20 µl of Proteinase K was added and incubated at 56 °C for 8 hours until the tissue was totally dissolved. Equal volume of PCI (25:24:1) was added and mixed thoroughly for few minutes. The sample was centrifuged for 10 minutes at 12,000 rpm. The upper phase was transferred to new 1.5 mL tube, equal volume of chloroform and iso-amyl alcohol (24:1) was added and centrifuged at 12,000 rpm for 10 minutes. The upper layer was transferred to a freshly sterilized micro centrifuge tube and double the volume of child absolute ethanol was added. This preparation was kept at -20 °C over night for precipitation, and centrifuged at 10,000 rpm for 10 minutes. The supernatant was discarded, then, 500 µl of 70% ethanol was added. The sample was again centrifuged at 7,000 rpm for 10 minutes and the supernatant was discarded. The pellet was kept for air dry under the laminar flow for some time, and re-suspended in 100 µl of nuclease free water or 1X TAE buffer (Sambrook *et al.,* 1989).

To the re-suspended sample, 500 µl of PCI was added and mixed slowly then incubated at 25 °C for 5 minutes and centrifuged at 12,000 rpm for 5 minutes at 4 °C. The aqueous phase was carefully removed into new centrifuge tube and treated two more times with PCI. The residual protein was eliminated from the aqueous phase by adding 400 µl of chloroform,

mixed slowly and centrifuged at 12,000 rpm for 10 minutes at 4 °C. The upper aqueous phase was recovered and the DNA was precipitated by adding 10 µl of 4 M ammonium acetate and 500 µl of cold absolute ethanol, then incubated at -20 °C for 20 minutes and centrifuged at 15,000 rpm for 15 minutes at 4 °C. The precipitated DNA was cleaned with ethanol and air dried. The pellet containing genomic DNA was dissolved in 100 µl of TE buffer and stored at -20 °C for future usage, or stored at -80 °C for long preservation.

Agarose gel electrophoresis (AGE)

Tank buffer (1X TAE) was prepared (365 mL; 350 mL, tank capacity and 15 mL, boat capacity). The presence of genomic DNA was confirmed through 1% agarose gel. Agarose (150 mg) was melted in 15 mL of TAE buffer under microwave oven for 1 minute. A drop of ethidium bromide was added and the gel was casted at room temperature. The polymerized gel along with the boat was fixed into the tank filled with 350 mL of 1X TAE buffer. The DNA sample was mixed with loading dye containing Bromophenol blue and Glycerol (2:6), and carefully loaded into the gel wells. The gel was supplied with 100 volt DC for 30 minutes and the DNA band was carefully documented.

Amplification of 16S r-RNA gene sequences

The 16S r-RNA gene sequences were amplification in Thermo Cycler (Applied Biosystem, Bengaluru, India, using the universal primers of forward and reverse in nature (5'-TGCCAGGCGGCCGCAGAGTRTGATCMTYGCTWAC-3', and 5'-TGCCAGGCGGCCGCCGYTAMCTTWTTACGRCT-3', respectively). PCR was carried out with a final reaction volume of 100 µl in thin walled PCR tube. The reaction mixture was in the following composition: DNA template (1 µl, 100 ng); forward and reverse primers (each 0.50 µl, 400 ng); 10X Chrom Taq RNA Polymerase Assay Buffer (10 µl); dNTPs (each 4 µl, 10 mM) in 4 Mm/$MgCl_2$; Chrom Taq RNA Polymerase Enzyme (1 µl, 3 U/µl); and DNase-RNase free water (75 µl). The PCR tubes containing the mixture were tapped gently and spun briefly at 10,000 rpm. The PCR tubes with all the components were transferred to thermal cycler. The thermo cycler condition was as follows: pre-running (initial denaturation) for 5 minutes at 90 °C; 35 cycles of final denaturation for 30 seconds each at 90 °C; annealing for 30 seconds at 50 °C; extension for 90 seconds at 72 °C; final extension for 7 minutes at 72 °C (Manufacturer's protocol, Chromous Biotech Pvt. Ltd. Bengaluru, India). To confirm the targeted PCR amplification, 4 µl of PCR product from each tube was mixed with 2 µl of 6X gel loading dye. The 2% agarose gel electrophoresis was constantly supplied with 50 V/cm for 20 min in 1X TAE buffer. The amplified product (the partial gene of 16S r-RNA) was visualized as a single compact band of expected size and carefully documented.

Sequencing reaction preparations

Sanger sequencing was done by outsourced with Chromous Biotech Pvt. Ltd. Bengaluru, India. As per manufacturer protocol, the partially amplified 16S r-RNA gene was denatured and annealed to an oligonucleotide primer, which was then extended by RNA polymerase using a mixture of deoxynucleotide triphosphates (normal dNTPs) and chain-terminating di-deoxynucleotide triphosphates (ddNTPs). The resulted newly synthesized RNA chain was with different length.

Template quantity for PCR product

The following are different template quantity, required to yield desired number of base pair sequences: 1-3 ng/µl to get 100-200 bp; 3-10 ng/µl to get 200-500 bp; 5-20 ng/µl for 500-1000 bp; 10-40 ng/µl for 1000-2000 bp; 25-50 ng/µl to get single stranded plasmid; and 150-300 ng/µl for double stranded plasmid. In this study, the desired number of base pairs was 1000-1500, and therefore they have used the template volume of 5-20 ng/µl.

Template pre-heat treatment

The template RNA was treated at 96 ºC for 5 minute in a Thermal Cycler, cold in ice bath immediately and stored at 4 ºC for further usage. The Big Dye Terminator kit (BDT) v 3.1 was thawed on ice and aliquot 10 µl of Ready Reaction (RR) mix into sterile 0.2 mL PCR thin walled microfuge tubes on ice. The reaction content was mixed briefly in tube and centrifuged for a quick spin of 20 seconds. The tubes were transferred to the PCR machine. The sequencing was performed with a total volume of 20 µl reaction mixture containing 3 µl of template DNA, 10 pM/µl of primers (forward, 0.50 µl and reverse, 0.50 µl), 6 µl of BDT-RR mix and 10 µl of Milli Q water. The PCR sequence cycling condition was as follows. 25 cycles of 60 seconds each at 96 °C for initial denaturation, which was followed by 25 cycles of 10 seconds each at 96 ºC for final denaturation, 25 cycles of 5 seconds each at 50 ºC for annealing (hybridization) and 25 cycles of 4 minutes each at 60 ºC for elongation (Manufacturer's protocol, Chromous Biotech Pvt. Ltd. Bengaluru, India).

Reactions clean-up by ethanolic precipitation

After completion of the PCR program, the sample was processed for ethanolic precipitation. From PCR tube, the samples were transferred to 96 well microlitre plates and 5 µl of 125 mM EDTA was added to each well. 60 µl of ice cold absolute ethanol (-20 ºC) was added to each reaction, the plate was sealed and mixed by vortexing for 20-30 seconds and incubated at room temperature for 15 minutes. The sample plate was spun at 3,000 × g for 30 minutes at 4 ºC. The supernatant was carefully removed by inverting the plate and spun up to 180 × g, then removed from the centrifuge. The pellet was rinsed once with 60 µl of ice cold 70% ethanol (-20 ºC) by centrifugation at 1650 × g for 15 minutes at 4 ºC. The plate was inverted and spun up to 180 × g for 1 minute, and then removed from the centrifuge. The sample was re-suspended in 10 µl of Hi-Di formamide and incubated for 15 minutes at room temperature. The re-suspended samples were transferred to appropriate wells of the sample plate and ensured that each sample was positioned at the bottom of the well. The samples were denatured at 95 ºC for 5 minutes with snap chill and the plate was loaded into sequencer and after completion, the data were analyzed.

Bioinformatics analysis

Sequence annotations

The sequence statistical analysis was conducted by various software's and online tools. The sequences were aligned with FASTA format, submitted to NCBI–GenBank database and authenticated. Before, the sequences were involved to find the nucleotide information, both forward and reverse sequences were merged (Contigs) with PRABI-Doua: CAP3 online tool. The sequences were subjected to basic local alignment search tool (BLAST) to find out the

internal stop codon and reading frame shift. Finally, the starting codon was found for detecting the translated protein by using ORF finder. Ban kit sequence submission tool was used to submit the sequence to GenBank.

Multiple sequence alignment

Multiple sequence alignment (MSA) was performed with *T*-Coffee package between three biological sequences, the protein, DNA and RNA of similar length. From the output, homology was inferred and the evolutionary relationship between the sequences was studied. The resulted sequences from *T*-coffee were uploaded in multiple align show (MAS) to highlight the amino acid residues in the sequences: Identical amino acid residues in amino acid colour, similar residues in black and variables in white. After selecting the parameters, the sequences were submitted and the result appeared in new window.

Results and discussion

Survival, growth, digestive enzymes and biochemical constituents

The SR and WG were found to significantly increase ($P<0.05$) in *B. subtilis* supplemented diets fed with *M. rosenbergii* when compared with control. Among different serially diluted concentrations of *B. subtilis*, 10^{-7} (CFU = 2.76×10^{-7}) produced the best performance. The FCR was found to correspondingly decrease in *B. subtilis* supplemented diets fed prawns. Therefore, *B. subtilis* supplemented diet was of quality and produced better growth and survival performances in *M. rosenbergii* (Table 1). The activities of digestive enzymes such as protease, amylase and lipase, and concentrations of basic biochemical constituents, such as total protein, amino acid, carbohydrate and lipid were also found to significantly increase, particularly at 2.76×10^{-7} CFU of *B. subtilis* supplemented diet fed with *M. rosenbergii* (Table 2).

Table 1. Nutritional indices of *M. rosenbergii* fed with *B. subtilis* supplemented diets (the initial length and weight were 2.86±0.05 cm and 0.39±0.05 g, respectively)

Parameter	Control	Serially diluted concentrations of *B. subtilis* (CFU)				
		5.87×10^{-1}	4.72×10^{-3}	3.13×10^{-5}	2.76×10^{-7}	1.11×10^{-9}
SR (%)	85.83±1.40^{a}	85.83±2.46^{a}	86.66±1.40^{a}	87.50±2.50^{a}	90.00±2.50^{a}	88.33±3.81^{a}
Length (cm)	3.90 ±0.20^{c}	4.20±0.20^{c}	4.40±0.30^{c}	5.20±0.30^{b}	5.80±0.40^{ab}	5.50±0.20^{b}
Weight (g)	0.78±0.15^{e}	1.40±0.12^{d}	1.60±0.15^{d}	2.30±0.14c	2.96±0.20^{b}	2.66±0.16^{a}
LG (cm)	1.10±0.20^{a}	1.30±0.20^{a}	1.50±0.30^{a}	2.30±0.30^{b}	2.90±0.40^{a}	2.60±0.20^{ab}
WG (g)	0.39±0.15^{d}	1.01±0.12^{c}	1.21±0.15^{c}	1.96±0.20^{b}	2.57±0.20^{a}	2.27±0.16^{b}
FCR (g)	8.58±3.35^{b}	2.99±0.36^{d}	2.50±0.31^{a}	1.53±0.17^{a}	1.18±0.11^{a}	1.32±0.09^{a}

Each value is mean ± standard deviation of three individual observations

Mean values within the same row sharing different alphabetical letter superscripts are statistically significant at $P<0.05$ (one-way ANOVA and subsequent post hoc multiple comparison with DMRT)

SR, survival rate; LG, length gain; WG, weight gain; FCR, food conversion ratio

Table 2. Activities of digestive enzymes (U/mg protein; *×10^2 U/mg protein) and concentrations of biochemical constituents (mg/g wet wt.) of *M. rosenbergii* fed with *B. subtilis* supplemented diets

Parameter	Control	Serially diluted concentrations of *B. subtilis* (CFU)				
		5.87×10^{-1}	4.72×10^{-3}	3.13×10^{-5}	2.76×10^{-7}	1.11×10^{-9}
Protease	3.73±0.20^{d}	4.53±0.14^{c}	4.64±0.15b^{c}	4.88±0.18^{b}	5.93±0.20^{a}	4.70±0.21^{bc}
Amylase	1.50±0.18^{e}	2.18±0.14^{d}	2.47±0.16^{d}	3.98±0.17^{b}	4.63±0.24^{a}	3.63±0.12^{c}
Lipase*	0.40±0.09^{e}	0.76±0.08^{d}	0.80±0.04^{cd}	0.98±0.05^{ab}	1.09±0.09^{a}	0.91±0.08^{bc}
Protein	80.13±3.11^{d}	85.72±3.85^{cd}	89.88±4.33^{bc}	94.96±4.15^{b}	113.50±5.27^{a}	106.70±4.57^{a}
Amino acid	29.22±1.51^{f}	43.32±2.23^{e}	50.22±2.58^{d}	67.45±2.72^{c}	73.34±3.85^{b}	57.65±3.65^{a}
Carbohydrate	18.17±1.15^{e}	19.13±1.72^{e}	24.47±1.75^{d}	33.03±1.84^{c}	37.47±1.87^{b}	28.37±1.64^{a}
Lipid	9.60±0.57^{d}	13.56±0.73^{c}	19.87±0.94^{b}	23.54±1.56^{a}	25.78±1.86^{a}	23.72±1.31^{a}

Each value is mean ± standard deviation of three individual observations.
Mean values within the same row sharing different alphabetical letter superscripts are statistically significant at $P<0.05$ (one-way ANOVA and subsequent post hoc multiple comparison with DMRT)

The significant increase in survival and growth performance with decrease in FCR, increases in activities of digestive enzymes and contents of basic biochemical constituents were also reported in *M. rosenbergii* fed with probiotics (*Lactobacillus* sp., *Bacillus licheniformis*, ® (*B. subtilis, B. coagulans, Lactobacillus sporogenes, Lactobacillus cremoris*, Biogen *Lactobacillus acidophilus, Lactobacillus plantarum*), LactoBacil®plus (*Bifidobacterium longum, Bifidobacterium bifidum, Lactobacillus acidophilus, Lactobacillus rhamnosus* and *Saccharomyces boulardii*), ViBact* (*B. mesentericus, C. butyricum*, *L. sporogenes* and *S. faecalis*), Binifit™ (*B. bifidum, Lactobacillus* sp*., L. acidophilus, Lactobacillus bulgaricus* and *Streptococcus thermophilus*), *Lactobacillus brevis, Lactobacillus fermentum*, *B. coagulans*, *B. subtilis*, *Enterococcus gallinarum* and *Enterococcus hirae* (Gupta *et al*., 2016; Jain *et al*., 2020a, b; Jayanthi *et al*., 2015a, b; Karthik and Bhavan, 2018; Karthik *et al*., 2018a, b; Kumar *et al*., 2013; Manjula *et al*., 2018; Narmatha *et al*., 2017; Saad *et al*., 2009; Seenivasan *et al*., 2011, 2012, 2013, 2014; Sudha *et al*., 2019; Suralikar and Sahu, 2001; Venkat *et al*., 2004).

Gut microbial consortium

Our previous study revealed the presence of *Streptococcus* sp., *Klepsiella* sp., *Escherichia coli,* and *Staphylococcus* sp., in the gut of *M. rosenbergii* control prawns (Manjula *et al*., 2018). In the present study, the gut of experimental prawns fed with *B. subtilis* supplemented diet showed the presence of two colonies of *Bacillus* spp., three colonies of *Lactobacillus* spp*.,* and a colony of *Streptococcus* sp., (Table 3). Competitive exclusion of pathogenic bacteria, *E. coli, Klebsiella* sp., and *Staphylococcus* sp. is indicated, because of supplementation of the probiotic bacterium, *B. subtilis*. Further, it is suggested that the commonsolistic bacterium, *B. subtilis* might have produced bacteriocins, proteinaceous toxins, which specifically inhibit members of pathogenic bacteria. Thus, in the absence of pathogenic bacteria, the survival and growth of *M. rosenbergii* was efficiently realized, due to maintenance of good general health.
The competitive exclusion of pathogenic bacteria, such as *Pseudomonas* spp, *Aeromonas* sp., *Klebsiella pneumonia*, *Acinetobacter* sp., *Salmonella* sp., *Staphylococcus* sp., C*itrobacter* sp., and *Streptococcus* sp., by probiotics like *B. subtilis*, *Lactobacillus* sp., *L. fermentum*, *L. brevis*,

B. coagulans, *B. licheniformis*, *E. gallinarum* and *E. hirae* has been reported in *M. rosenbergii* (Bhavan, 2018; Dash *et al.,* 2014; Jain *et al*., 2020a,b; Jayanthi *et al*., 2015a, b; Karthik *et al*., 2018a, b; Manjula *et al*., 2018; Narmatha *et al*., 2017; Sudha *et al*., 2019a; Venkat *et al*., 2004). Therefore, probiotics play an important role in welfare of the host by maintaining a healthier balance of intestinal microflora, which not only provides a defensive barrier against colonization of harmful bacteria but also stimulates the immune system of the host (Cain and Swan, 2010; Gomez *et al.,* 2008). Table 4 depicts the confirmative results of various tests conducted for the bacteria detected in the gut of *M. rosenbergii* PL**.**

Table 3. Microbial load in the gut of *M. rosenbergii* PL fed with *B. subtilis* (CFU=2.76×10^{-7}) supplemented diet

Samples	Isolated species	Composition (%)	CFU
Control (Manjula *et al*., 2018)	*Streptococcus* sp.,	18.48	34.55×10^{-4}
	Klebsiella sp.,	18.10	25.20×10^{-4}
	Escherichia coli	22.62	20.55×10^{-4}
	Staphylococcus sp.,	26.25	17.20×10^{-4}
B. subtilis (2.76×10^{-7})	*Bacillus* spp.,	38.25	36.45×10^{-4}
	Lactobacillus spp.,	32.81	25.02×10^{-4}
	Streptococcus sp.,	23.02	32.20×10^{-4}

CFU, Colony forming units

Table 4. Confirmative results of biochemical tests for microflora present in the gut of *M. rosenbergii* PL fed with *B. subtilis* supplemented diet

Test	Gut of control PL (our previous study, Manjula *et al*., 2018)				Gut of experimental PL (the present study)		
	St sp.,	K sp.,	Ec	Sta sp.,	Ba spp.,	La spp.,	St sp.,
Gram's staining	+	+	-	+	+	+	+
Motility test	+	+	+	+	+	+	+
Indole test	-	+	+	-	-	-	-
Methyl red test	-	+	+	-	-	-	-
Voges-Proskauer test	+	-	+	+	-	-	+
Citrate utilization test	+	-	-	+	+	-	+
Starch hydrolases	+	-	+	+	+	-	+
Gelatin hydrolases	+	-	-	+	+	-	+
Nitrate reduction test	+	+	+	+	+	-	+
Oxidase test	-	-	+	-	-	-	-
Catalase test	A	A	A	A	+	A	A
Glucose test	A	A	NA	A	A	A	A
Lactose test	A	A	A	A	A	A	A
Sucrose test	A	A	A	A	A	A	A
Manitol test	A	A	A	A	A	A	A
Maltose test	NA	A	Na	NA	A	A	NA

+, Positive; -, Negative; A, Acid production; NA, No acid production.
St sp., *Streptococcus* sp.; K sp., *Klebsiella* sp.,; Ec, *Escherichia coli*; Sta sp., *Staphylococcus* sp.,; Ba spp., *Bacillus* spp.; La spp, *Lactobacillus* spp.

Molecular characterisation

The genomic DNA of different bacterial colonies harvested in the gut of *M. rosenbergii* PL fed with *B. subtilis* supplemented diet showed greater size than 10 kb sized nucleotides (Figure 3) for *B. subtilis*, *B. cereus*, *L. delbrueckii*, *L. casei*, *L. rhamnosus* and *S. pyogens*. The PCR amplified product of 16S r-RNA gene partial sequences of these bacterial species showed greater than 1500 bp sized nucleotides. Actually the aligned sequences showed 1485 bp, 1490 bp, 1519 bp, 1420 bp, 1408 bp and 1493 bp for *B. subtilis*, *B. cereus*, *L. delbrueckii*, *L. casei*, *L. rhamnosus* and *S. pyogens*, respectively (Figure 4). The NCBI GenBank accession number assigned for these sequences and sequence similarity (99-100%) available with NCBI database are presented in Table 5.

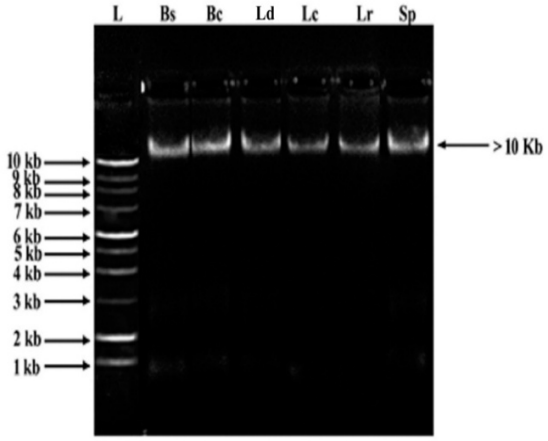

Figure 3. AGE (1%) of genomic DNA (>10 kb) isolated from various bacterial species grown in the gut of *M. rosenbergii* fed with *B. subtilis* supplemented diet.
L, Ladder (1 kb); **Bs**, *Bacillus subtilis*; **Bc**, *Bacillus cereus*; **Ld**, *Lactobacillus delbrueckii*; **Lc**, *Lactobacillus casei*; **Lr**, *Lactobacillus rhamnosus*; **Sp**, *Streptococcus pyogens*.

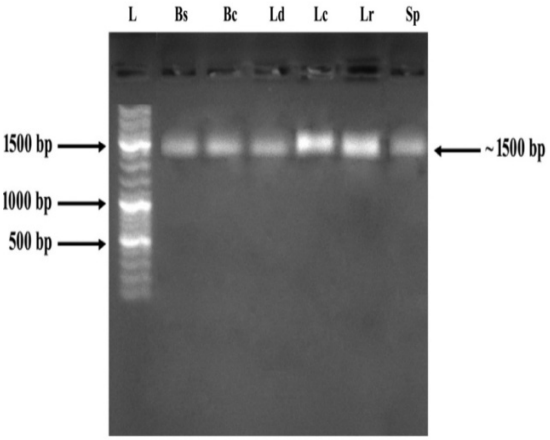

Figure 4. AGE (2%) of amplified products (~1500 bp) of 16S r-RNA gene of various bacterial species grown in the gut of *M. rosenbergii* fed with *B. subtilis* supplemented diet.
L, Ladder (100 bp); **Bs**, *Bacillus subtilis*; **Bc**, *Bacillus cereus*; **Ld**, *Lactobacillus delbrueckii*; **Lc**, *Lactobacillus casei*; **Lr**, *Lactobacillus rhamnosus*; **Sp**, *Streptococcus pyogens*.

Table 5. BLAST identification of 16S r-RNA gene sequences of subjected and retrieved bacteria species

Subjected sequences with paper author's accession number	Identical (%)	Retrieved/ matched sequences with accession number
Bacillus subtilis MG557832	99	*Bacillus subtilis* NR027552 (USA), Sproeer, 2015
Bacillus cereus MG557833	100	*Bacillus cereus* NR074540 (USA), Ivanova *et al*., 2013
Lactobacillus delbrueckii MG557834	99	*Lactobacillus bulgaricus* NR075019 (USA), van de Guchte *et al.*,2013
Lactobacillus casei MG557835	100	*Lactobacillus casei* NR113333 (USA), Miyashita and Nakagawa, 2011
Lactobacillus rhamnosus MG557836	100	*Lactobacillus rhamnosus* KM096587 (India), Wagh Deshpande, 2014
Streptococcus pyogenes MG557837	100	*Streptococcus pyogenes* EU660342 (India), Thenmozhi, *et al*., 2008

The results of multiple sequence alignment showed 1029 identical amino acids residues, 77 similar amino acids residues and 394 variable amino acids sites. These data revealed more identical amino acid residues, which indicate the fact that the species were closely related with each other, because of *Bacillus* and *Lactobacillus* spp., alone (Figure 5). However, the base compositions varied among the species; AT biases were ranged from 45.0% to 47.7% (*Bacillus subtilis* and *Lactobacillus rhamnosus*, respectively). Similarly, the GC biases ranged from 52.3% to 55.00% (*Lactobacillus rhamnosus* and *Bacillus subtilis*, respectively) (Table 6). The lower AT bias recorded indicates the fact that the less abundance of nuclear copies of mt-DNA (NUMTs) genes is known as pseudogenes, homologs or paralogs, because of closely related species.

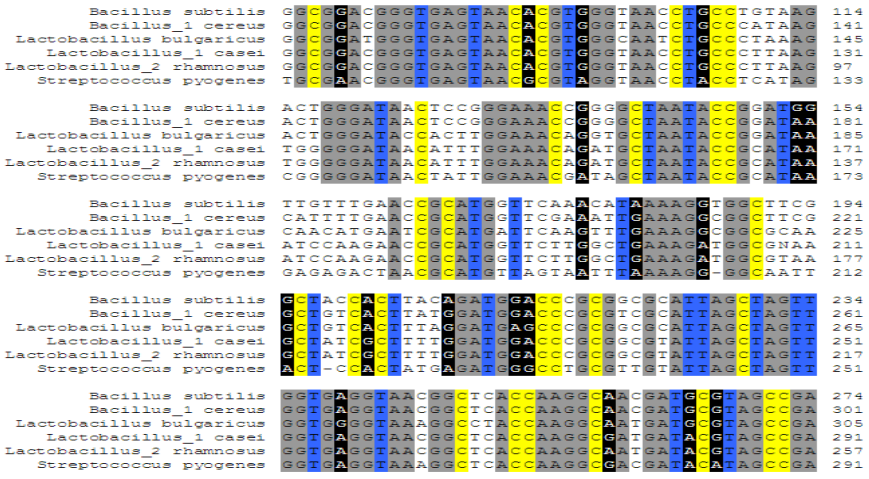

Figure 5. Multiple sequence alignment of 16S r-RNA gene sequences of bacterial species identified in the gut of *M. rosenbergii* PL fed with *B. subtilis* supplemented diet. An alignment is formatted by using multiple align show (MAS) with coloured background and a consensus setting of 100%. Identical residues are indicated by amino acid colour and similar residues are black in colour. Gaps and other residues are given in white background

Table 6. Nucleotide composition of bacterial species identified through 16S r-RNA gene sequences in the gut of *M. rosenbergii* PL fed with *B. subtilis* incorporated diet

Species	Nucleotide (%)					
	A	T	AT	G	C	GC
Bacillus subtilis	25.2	19.8	45.0	31.5	23.5	55.0
Bacillus cereus	25.6	21.1	46.6	30.7	22.7	53.4
Lactobacillus delbrueckii	25.7	20.9	46.5	30.9	22.6	53.5
Lactobacillus casei	25.7	21.5	47.2	30.3	22.6	52.8
Lactobacillus rhamnosus	25.6	22.1	47.7	29.9	22.4	52.3
Streptococcus pyogenes	26.1	21.2	47.3	30.5	22.2	52.7
Average	25.6	21.1	46.7	30.6	22.6	53.3

Nucleotide divergence

The mean divergent rate among different subjected bacterial species ranged from 1.092 to 3.655 (*B. subtilis* Vs. *B. cereus*, and *B. cereus* Vs. *S. pyogenes*, respectively) with an average of 1.688. The divergent rate was >3% between *B. subtilis* and *B. cereus*, which indicates the fact that these species are distinct, whereas other species are closely related (Table 7). According to (Engene and Herwick, 2011), a high degree of intra-genomic variation in 16S r-RNA gene copies has been reported in *Lactobacillus rhamnosus* (0–7.67%), *Caldanaerobacter subterraneus* (0.03–6.23%), *Desulfitobacterium hafniense* (0.06–3.73%), *Bacteroides ovatus* (0.07–3.30%), *Yersinia enterocolitica* (0–2.67%) and *Desulfitobacterium dehalogenans* (0–2.14%). (Pei *et al.*, 2010) reported that *E. coli* is known to have high intra-specific variation of 1.10% between multiple 16S r-RNA genes in the genome.

Table 7. Nucleotide divergence of bacterial species identified in the gut of *M. rosenbergii* PL fed with *B. subtilis* supplemented diet

Between Species	Divergence (%)
Bacillus subtilis Vs. *Bacillus cereus*	3.655
Bacillus subtilis Vs. *Lactobacillus delbrueckii*	1.831
Bacillus cereus Vs. *Lactobacillus delbrueckii*	1.520
Bacillus subtilis Vs. *Lactobacillus casei*	2.382
Bacillus cereus Vs. *Lactobacillus casei*	1.505
Lactobacillus bulgaricus Vs. *Lactobacillus casei*	1.313
Bacillus subtilis Vs. *Lactobacillus rhamnosus*	2.382
Bacillus cereus Vs. *Lactobacillus rhamnosus*	1.505
Lactobacillus delbrueckii Vs. *Lactobacillus rhamnosus*	1.313
Lactobacillus casei Vs. *Lactobacillus rhamnosus*	1.123
Bacillus subtilis Vs. *Streptococcus pyogenes*	1.092
Bacillus cereus Vs. *Streptococcus pyogenes*	1.092
Lactobacillus delbrueckii Vs. *Streptococcus pyogenes*	1.542
Lactobacillus casei Vs. *Streptococcus pyogenes*	1.260
Lactobacillus rhamnosus vs. *Streptococcus pyogenes*	1.308
Average	1.688

The molecular characterization of gut bacterial consortium of *M. rosenbergii* fed with dietary supplementations of probiotic products and bacteria revealed that LactoBacil®$_{plus}$ and ViBact* have eliminated *Pseudomonas* sp., *L. brevis* and *L. fermentum* have excluded *K. pneumonia*, *B. coagulans* has competitively excluded *Streptococcus* sp., and *Klebsiella* sp., *B. licheniformis* has competitively eliminated *Pesudomonas* sp., *Klebsiella* sp., *Staphylococcus* sp., and *Acinetobacter* sp., from the gut of experimental prawns (Karthik and Bhavan, 2018; Karthik *et al*., 2018b; Jayanthi *et al*., 2015a, b; Manjula *et al*., 2018, 2019; Sudha *et al*., 2019a, b).

Conclusions

The probiotic bacterium, *B. subtilis*, particularly at 10^{-7} (CFU=$2.76x10^{-7}$) concentration has produced the best survival and growth of *M. rosenbergii*, and competitively excluded *E. coli*, *Klebsiella* sp., and *Staphylococcus* sp. It is suggested that this probiotic bacterium possesses immunomodulatory effect and improves the general health of the prawn due to maintenance of good intestinal health because of colony establishment of, particularly *B. subtilis* and few *Lactobacillus* spp. The increases recorded in activities of digestive enzymes indicate the fact that the macromolecules of protein, carbohydrate and fat were well digested, absorbed and assimilated. Therefore, concentrations of total protein, amino acid, carbohydrate and lipid were elevated in prawn fed with *B. subtilis* supplemented diet, which in turn ultimately produced the best growth and survival of *M. rosenbergii*. The pathogenic bacteria, such as *Escherichia coli*, *Klebsiella* spp., and *Staphylococcus* spp., present in the control prawns as per our previous report (Manjula *et al*., 2018), have competitively been excluded by the experimental prawns fed with *B. subtilis* supplemented diet in the present study. Thus, *B. subtilis* is recommended as a feed additive for sustainable culture of scampi.

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