



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

Periakali Saravana Bhavan*, Duraisamy Anitha, Thankaraj Manjula, Rajendran Udayasuriyan, Ramasamy Kalpana, Madhayan Karthik

Department of Zoology, Bharathiar University, Coimbatore-641046, Tamil Nadu, India

*Corresponding Author E-mail: bhavan@buc.edu.in

Received: 27 April 2020, Revised: 17 June 2020, Accepted: 9 July 2020

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, phenylalanine 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 administered 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* B119, 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.

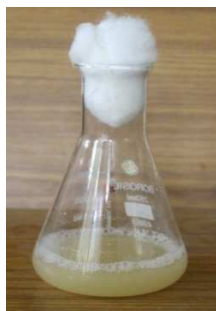


Figure 1. Culture morphology of *Bacillus subtilis* in nutrient broth



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 feed 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 (DO₂ (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 °C 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 °C 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 °C. Then, 0.1 mL of broth culture was seeded over the surface of freshly prepared nutrient agar plate and incubates at 37 °C 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 cold 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 amplified in Thermo Cycler (Applied Biosystem, Bengaluru, India, using the universal primers of forward and reverse in nature (5'-TGCCAGGCGGCCGAGAGTRTGATCMTYGCTWAC-3', and 5'-TGCCAGGCGGCCGCGYTAMCTTWTACGRCT-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/MgCl₂; 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/ μ l to get 100-200 bp; 3-10 ng/ μ l to get 200-500 bp; 5-20 ng/ μ l for 500-1000 bp; 10-40 ng/ μ l for 1000-2000 bp; 25-50 ng/ μ l to get single stranded plasmid; and 150-300 ng/ μ l 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/ μ l.

Template pre-heat treatment

The template RNA was treated at 96 °C for 5 minute in a Thermal Cycler, cold in ice bath immediately and stored at 4 °C for further usage. The Big Dye Terminator kit (BDT) v 3.1 was thawed on ice and aliquot 10 μ l 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 μ l reaction mixture containing 3 μ l of template DNA, 10 pM/ μ l of primers (forward, 0.50 μ l and reverse, 0.50 μ l), 6 μ l of BDT-RR mix and 10 μ l 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 °C for final denaturation, 25 cycles of 5 seconds each at 50 °C for annealing (hybridization) and 25 cycles of 4 minutes each at 60 °C 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 μ l of 125 mM EDTA was added to each well. 60 μ l of ice cold absolute ethanol (-20 °C) 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 \times g for 30 minutes at 4 °C. The supernatant was carefully removed by inverting the plate and spun up to 180 \times g, then removed from the centrifuge. The pellet was rinsed once with 60 μ l of ice cold 70% ethanol (-20 °C) by centrifugation at 1650 \times g for 15 minutes at 4 °C. The plate was inverted and spun up to 180 \times g for 1 minute, and then removed from the centrifuge. The sample was re-suspended in 10 μ l 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 °C 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 <i>B. subtilis</i> (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 ± 0.14^c	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 \pm 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; * $\times 10^2$ 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 <i>B. subtilis</i> (CFU)				
		5.87×10^{-1}	4.72×10^{-3}	3.13×10^{-5}	2.76×10^{-7}	1.11×10^{-9}
Protease	3.73 \pm 0.20 ^d	4.53 \pm 0.14 ^c	4.64 \pm 0.15 ^{bc}	4.88 \pm 0.18 ^b	5.93 \pm 0.20 ^a	4.70 \pm 0.21 ^{bc}
Amylase	1.50 \pm 0.18 ^e	2.18 \pm 0.14 ^d	2.47 \pm 0.16 ^d	3.98 \pm 0.17 ^b	4.63 \pm 0.24 ^a	3.63 \pm 0.12 ^c
Lipase*	0.40 \pm 0.09 ^e	0.76 \pm 0.08 ^d	0.80 \pm 0.04 ^{cd}	0.98 \pm 0.05 ^{ab}	1.09 \pm 0.09 ^a	0.91 \pm 0.08 ^{bc}
Protein	80.13 \pm 3.11 ^d	85.72 \pm 3.85 ^{cd}	89.88 \pm 4.33 ^{bc}	94.96 \pm 4.15 ^b	113.50 \pm 5.27 ^a	106.70 \pm 4.57 ^a
Amino acid	29.22 \pm 1.51 ^f	43.32 \pm 2.23 ^e	50.22 \pm 2.58 ^d	67.45 \pm 2.72 ^c	73.34 \pm 3.85 ^b	57.65 \pm 3.65 ^a
Carbohydrate	18.17 \pm 1.15 ^e	19.13 \pm 1.72 ^e	24.47 \pm 1.75 ^d	33.03 \pm 1.84 ^c	37.47 \pm 1.87 ^b	28.37 \pm 1.64 ^a
Lipid	9.60 \pm 0.57 ^d	13.56 \pm 0.73 ^c	19.87 \pm 0.94 ^b	23.54 \pm 1.56 ^a	25.78 \pm 1.86 ^a	23.72 \pm 1.31 ^a

Each value is mean \pm 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., *Klebsiella* 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., *Citrobacter* 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)	<i>Streptococcus</i> sp.,	18.48	34.55×10 ⁻⁴
	<i>Klebsiella</i> sp.,	18.10	25.20×10 ⁻⁴
	<i>Escherichia coli</i>	22.62	20.55×10 ⁻⁴
	<i>Staphylococcus</i> sp.,	26.25	17.20×10 ⁻⁴
<i>B. subtilis</i> (2.76×10 ⁻⁷)	<i>Bacillus</i> spp.,	38.25	36.45×10 ⁻⁴
	<i>Lactobacillus</i> spp.,	32.81	25.02×10 ⁻⁴
	<i>Streptococcus</i> 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. pyogenes*. 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. pyogenes*, 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.

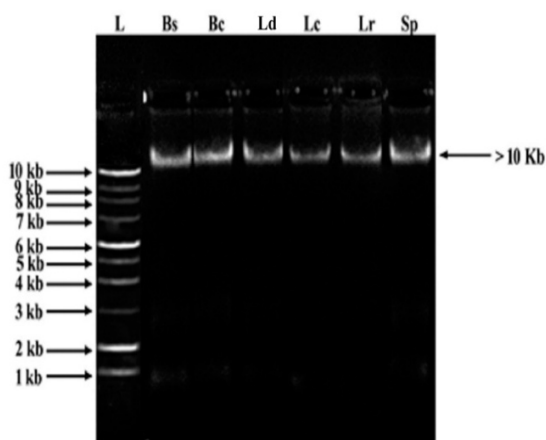


Figure 3. AGE (1%) of genomic DNA (>10 kb) isolated from various bacterial species grown in the gut of *M. rosenbergii* fed with *B. subtilis* supplemented diet. **L**, Ladder (1 kb); **Bs**, *Bacillus subtilis*; **Bc**, *Bacillus cereus*; **Ld**, *Lactobacillus delbrueckii*; **Lc**, *Lactobacillus casei*;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

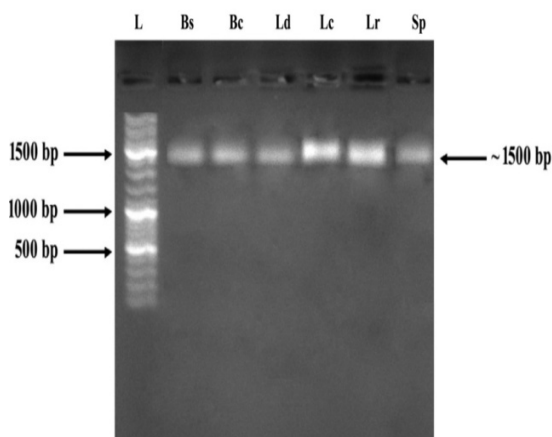


Figure 4. AGE (2%) of amplified products (~1500 bp) of 16S r-RNA gene of various bacterial species grown in the gut of *M. rosenbergii* fed with *B. subtilis* supplemented diet. **L**, Ladder (100 bp); **Bs**, *Bacillus subtilis*; **Bc**, *Bacillus cereus*; **Ld**, *Lactobacillus delbrueckii*; **Lc**,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

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
<i>Bacillus subtilis</i> MG557832	99	<i>Bacillus subtilis</i> NR027552 (USA), Sproeer, 2015
<i>Bacillus cereus</i> MG557833	100	<i>Bacillus cereus</i> NR074540 (USA), Ivanova et al., 2013
<i>Lactobacillus delbrueckii</i> MG557834	99	<i>Lactobacillus bulgaricus</i> NR075019 (USA), van de Guchte et al., 2013
<i>Lactobacillus casei</i> MG557835	100	<i>Lactobacillus casei</i> NR113333 (USA), Miyashita and Nakagawa, 2011
<i>Lactobacillus rhamnosus</i> MG557836	100	<i>Lactobacillus rhamnosus</i> KM096587 (India), Wagh Deshpande, 2014
<i>Streptococcus pyogenes</i> MG557837	100	<i>Streptococcus pyogenes</i> 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.

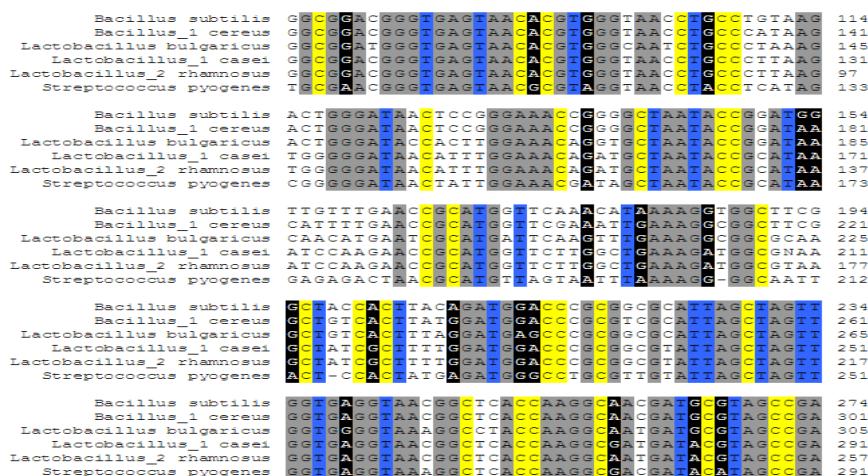


Figure 5. Multiple sequence alignment of 16S r-RNA gene sequences of bacterial species identified in the gut of *M. rosenbergii* PL fed with *B. subtilis* supplemented diet. An alignment is formatted by using multiple align show (MAS) with coloured background and a consensus setting of 100%. Identical 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
<i>Bacillus subtilis</i>	25.2	19.8	45.0	31.5	23.5	55.0
<i>Bacillus cereus</i>	25.6	21.1	46.6	30.7	22.7	53.4
<i>Lactobacillus delbrueckii</i>	25.7	20.9	46.5	30.9	22.6	53.5
<i>Lactobacillus casei</i>	25.7	21.5	47.2	30.3	22.6	52.8
<i>Lactobacillus rhamnosus</i>	25.6	22.1	47.7	29.9	22.4	52.3
<i>Streptococcus pyogenes</i>	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 (%)
<i>Bacillus subtilis</i> Vs. <i>Bacillus cereus</i>	3.655
<i>Bacillus subtilis</i> Vs. <i>Lactobacillus delbrueckii</i>	1.831
<i>Bacillus cereus</i> Vs. <i>Lactobacillus delbrueckii</i>	1.520
<i>Bacillus subtilis</i> Vs. <i>Lactobacillus casei</i>	2.382
<i>Bacillus cereus</i> Vs. <i>Lactobacillus casei</i>	1.505
<i>Lactobacillus bulgaricus</i> Vs. <i>Lactobacillus casei</i>	1.313
<i>Bacillus subtilis</i> Vs. <i>Lactobacillus rhamnosus</i>	2.382
<i>Bacillus cereus</i> Vs. <i>Lactobacillus rhamnosus</i>	1.505
<i>Lactobacillus delbrueckii</i> Vs. <i>Lactobacillus rhamnosus</i>	1.313
<i>Lactobacillus casei</i> Vs. <i>Lactobacillus rhamnosus</i>	1.123
<i>Bacillus subtilis</i> Vs. <i>Streptococcus pyogenes</i>	1.092
<i>Bacillus cereus</i> Vs. <i>Streptococcus pyogenes</i>	1.092
<i>Lactobacillus delbrueckii</i> Vs. <i>Streptococcus pyogenes</i>	1.542
<i>Lactobacillus casei</i> Vs. <i>Streptococcus pyogenes</i>	1.260
<i>Lactobacillus rhamnosus</i> vs. <i>Streptococcus pyogenes</i>	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 *Pseudomonas* 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.

Acknowledgements

The authors thank M/S. Chromous Biotech Pvt., Ltd., Bengaluru, India, for outsourcing services provided.

Conflict of interest: The authors declare that they have no competing interests.

References

- Agrebi, R, Haddar, A, Hajji, M, Frikha, F, Manni, L, Jellouli, K, Nasri, M. (2009). Fibrinolytic enzymes from a newly isolated marine bacterium *Bacillus subtilis* A26: characterization and statistical media optimization. *Can. J. Microbiol.*, 55:1049-1061.
- Aly, SM. (2009). *Probiotics and Aquaculture*. CAB Reviews: Perspectives in Agriculture, Veterinary Science, Nutrition and Natural Resources, 4(074), pp. 1-16.
- AOAC. (2005). Association of Official Analytical Chemists; Official Methods of Analysis. 18th edn. Arlington, VA, USA.
- APHA. (2005). American Public Health Association. Standard Methods for the Examination of Water and Waste Water. Method 2540. 21st Edition.

- Balaji, K, Sahu, NP, Tripathi, SD. (2002). Effect of different protein energy ratio in isocaloric diet on growth and survival of post larvae of *Macrobrachium rosenbergii* (De Man). Aquaculture Nutrition Workshop Proceeding, pp. 7-10.
- Balazs, GH, Ross, E. (1976). Effect of protein source and level on growth and performance of the captive freshwater prawn, *Macrobrachium rosenbergii*. *Aquaculture*, 7:229–313.
- Barnes, H, Blackstock, J. (1973). Estimation of lipids in marine animals and tissues. Detail investigation of the sulpho-phosphovanillin method for total lipids. *J. Exp. Mar. Biol. Ecol.*, 12:103–118.
- Bernfeld, P. (1955) Amylases, alpha and beta. In: Colowick, S. P. and Kaplan, N. O (Eds.). *Methods in enzymology*, New York: Academic Press, 1:149–158. [http://dx.doi.org/10.1016/0076-6879\(55\)01021-5](http://dx.doi.org/10.1016/0076-6879(55)01021-5).
- Bidhan, CD, Meena, DK, Behera, BK, Das, P, Mohapatra, PKD, Sharma, AP. (2014). Probiotics in fish and shellfish culture: immunomodulatory and ecophysiological responses. *Fish Physiol. Biochem.*, 40:921–971. DOI 10.1007/s10695-013-9897-0.
- Brown, JH. (1991). Freshwater prawns. In: *Production of Aquatic Animals*, World Animal Science, C4, (Ed. by C.E. Nash), Elsevier Scientific Publishing, Amsterdam. pp. 31-43.
- Cain, K, Swan, C. (2010). Barrier function and immunology. In: *the multifunctional gut of fish* (ed. by M. Grosell, A. P. Farrell & C. J. Brauner). Academic Press, Amsterdam. pp. 111-134.
- Chunchom, S, Deeseenthum, S, Kongbanchad, W, Pakdeenarong, N. (2010). Culturing of the giant freshwater prawns fed with Thai fairy Shrimp, *Branchinella thailandensis*. *J. Microscopy Soc. Thailand*, 24:9-12.
- Cruz, PM, Ibanez, AL, Hermosillo, OAM, Saad HCR. (2012). Use of Probiotics in Aquaculture. *ISRN Microbiology*, doi:10.5402/2012/916845 .
- Das, NN, Saad, CR, Ang, KJ, Law, AT, Harmin, SA. (1996). Diet formulation for *Macrobrachium rosenbergii* (de Man) broodstock based on essential amino acid profile of its egg. *Aquacult. Res.*, 27(7):543-555.
- Dash, BK, Rahman, MM, Sarker, PK. (2015). Molecular Identification of a Newly Isolated *Bacillus subtilis* BI19 and Optimization of Production Conditions for Enhanced Production of Extracellular Amylase. *BioMed Res. Int.*, Article ID 859805, 9 pages <http://dx.doi.org/10.1155/2015/859805>.
- Fadul, AE, Obeid, E, Alawad, AM, Ibrahim, HM. (2015). Isolation and Characterization of *Bacillus Subtillus* with Potential Production of Nattokinase. *Int. J. Adv. Res.*, 3(3):94-101.
- Folch, J, Lees, M, Bloane-Stanley, GH. (1957). A simple method for the isolation and purification of total lipids from animal tissues. *J. Biol. Chem.*, 266:497–509.

Furne, M, Hidalgo, MC, Lopez, A, Garcia-Gallego, M, Morales, AE, Domezain, A, Domezaine, J, Sanz, A. (2005). Digestive enzyme activities in Adriatic sturgeon *Acipenser naccarii* and rainbow trout *Oncorhynchus mykiss*. A comparative study. *Aquaculture*, 250:391-398.

Ghosh, K, Sen, SK, Ray, AK. (2004). Growth and survival of rohu *Labeo rohita* (Hamilton) spawn fed diets fermented with intestinal bacterium, *Bacillus circulans*. *Acta Ichthyol. Et Piscat.*, 34:155-165.

Ghosh, S, Sinha, A, Sahu, C. (2008). Dietary probiotic supplementation on growth and health of live-bearing ornamental fishes. *Aquac. Nutr.*, 14(4):289-299.

Gomez, R, Geovanny, D, Shen, MA. (2008). Influence of probiotics on the growth and digestive enzyme activity of white Pacific shrimp (*Litopenaeus vannamei*). *Oceanic Coastal Sea Res.*, 7:215-218.

Haroun, EREL, Goda, AMAS, Chowdhury, MAK. (2006). Effect of dietary probiotic Biogen® supplementation as a growth promoter on growth performance and feed utilization of Nile tilapia *Oreochromis niloticus* (L.). *Aquac. Res.*, 37:1473-1480.

Holt, JG, Krieg, NR, Sneath, PHA, Staley, JT, Williams, ST. (1996). *Bergey's Manual of Determination Bacteriology*, 9th Edition, Williams and Wilkins, Baltimore, (MD).

Holzappel, WH, Haberer, P, Geisen, R, Björkroth, J, Schillinger, U. (2001). Taxonomy and important features of probiotic microorganisms in food and nutrition. *Am. J. Clin. Nutr.*, 73(suppl):365S-373S.

Holzappel, WH, Haberer, P, Snel, J, Schillinger, U, Jos, HJ, Veld, H. (1998). Overview of gut flora and probiotics. *Int. J. Food Microbiol.*, 41:85-101.

Hoseinifar, SH, Sun, YZ, Wang, A, Zhou, Z. (2018). Probiotics as Means of Diseases Control in Aquaculture, a Review of Current Knowledge and Future Perspectives. *Front. Microbiol.*, <https://doi.org/10.3389/fmicb.2018.02429>.

Jain, IB, Bhavan, PS, Kalpana, R, Manjula, T, Dharani, C, Muralisankar, T, Veerapandi, A, Karthik, M. (2020a). Performance of probiotic bacterium, *Enterococcus gallinarum* enriched *Artemia franciscana* nauplii on survival, growth and basic biochemical constituents of the prawn *Macrobrachium rosenbergii* post larvae. *Schol. Aca. J. Biosci.*, 8(2):13-22.

Jain, IB, Bhavan, PS, Manjula, T, Dharani, C, Kalpana, R, Muralisankar, T, Aiswaryalakshmi, S, Karthik, M. (2020b). Growth Performance of the Prawn *Macrobrachium rosenbergii* Post Larvae Fed with Probiotic Bacterium, *Enterococcus hirae* Enriched *Artemia franciscana* Nauplii. *Int. J. Zool. Invest.*, 6(1):107-121.

Jayanthi, L, Bhavan, PS, Srinivasan, V, Muralisankar, T, Manickam, N. (2015a). Probiotics product (LactoBacil®_{plus}) on improvement of survival, growth, digestive enzymes activity, nutritional status and gut microflora of the prawn *Macrobrachium rosenbergii*. *Int. J. Curr. Res.*, 7:11440-11453.

Jayanthi, L, Bhavan, PS, Srinivasan, V, Muralisankar, T, Manickam, N. (2015b). Dietary supplementation of probiotics product (ViBact*) on the survival, growth, biochemical constituents and gut microflora of the giant freshwater prawn *Macrobrachium rosenbergii* post-larvae. *Asian J. Biochem. Pharm. Res.*, 2:67–88.

Karthik, M, Bhavan, PS, Manjula, T. (2018a). Growth Promoting Potential and Colonization Ability of Probiotics (*Bacillus coagulans* and *Bacillus subtilis*) on the Freshwater Prawn *Macrobrachium rosenbergii* Post-Larvae. *Insights Biol. Med.*, 2:007–018.

Karthik, M, Bhavan, PS, Seenivasan, V, Asaikkutti, A, Muralisankar, T, Mahendran, R. (2018b). Dietary Supplementation of *Lactobacillus fermentum* for Improving the Survival, Growth and Nutritional Profiles of the Prawn *Macrobrachium rosenbergii*, and 16S rDNA based Identification of its Establishment. *Scholar Rep.*, V3-I1-06:38–62.

Karthik, M, Bhavan, PS. (2018). Supplementation of *Lactobacillus brevis* for Growth Promotion of the Freshwater Prawn *Macrobrachium rosenbergii* Post Larvae and identification of Gut Microflora through 16s r-DNA. *Res. J. Biotech.*, 13(1):34–50.

Kavitha, M, Raja, M, Perumal, P. (2018). Evaluation of probiotic potential of *Bacillus* spp. isolated from the digestive tract of freshwater fish *Labeo calbasu* (Hamilton, 1822). *Aquacul. Rep.*, 11:59-69.

Kumar, NR, Raman, RP, Jadhao, SB, Brahmchari, RK, Kumar, K, Dash, G. (2013). Effect of dietary supplementation of *Bacillus licheniformis* on gut microbiota, growth and immune response in giant freshwater prawn, *Macrobrachium rosenbergii* (de Man, 1879). *Aquacul. Int.*, 21(2):387–403.

Kumar, R, Mukherjee, SC, Prasad, KP, Pal, AK. (2006). Evaluation of *Bacillus subtilis* as a probiotic to Indian major carp *Labeo rohita* (Ham.). *Aquacul. Res.*, 37:1215-1221.

Li, J, Xu, Y, Jin, L, Li, X. (2015). Effects of a probiotic mixture (*Bacillus subtilis* YB-1 and *Bacillus cereus* YB-2) on disease resistance and non-specific immunity of sea cucumber, *Apostichopus japonicus* (Selenka). *Aquacul. Res.*, 46:3008–3019.

Lowry, OH, Rosenbrough, WJ, Fair, AL, Randall, RJ. (1951). Protein measurement with the Folin phenol reagent. *J. Biol. Chem.*, 193:265–275.

Manjula, S, Bhavan, PS, Karthik, M, Anitha, D, Kalpana, R, Manjula, T. (2018). Survival, Growth, Activities of Digestive Enzymes, Concentrations of Basic Biochemical Constituents and Competitive Exclusion of Pathogenic Bacteria in *Bacillus coagulans* Supplemented Diet Fed *Macrobrachium rosenbergii* Post-Larvae. *Int. J. Res. Stud. Sci. Eng. Technol.*, 5(12):9-22.

Manjula, S, Bhavan, PS, Udayasuriyan, R, Anitha, D, Kalpana, R, Manjula, T. (2019). Molecular identification of gut microflora of *Bacillus coagulans* supplemented feed fed *Macrobrachium rosenbergii* post-larvae using 16S rRNA. *Int. J. Biotech.*, 8(1):19-37.

- Millamena, OM., Teruel, MB., Kanazawa, A, Teshima, S. (1999). Quantitative dietary requirements of postlarval tiger shrimp, *Penaeus monodon*, for histidine, isoleucine, leucine, phenylalanine and tryptophan. *Aquaculture*, 179:169–179.
- Mitra, G, Mukopadhyay, PK, Chattopadhyay, DN. (2005). Nutrition and feeding in freshwater prawn (*Macrobrachium rosenbergii*) farming. *Aquatic Feeds: Formulats. Beyond*, 2:17–19.
- Moore, S, Stein, WH. (1948). Photometris Ninhydrin Method for Use in the Chromatography of Amino Acids. *J. Biol. Chem.*, 176:367–388.
- Mukherjee, AK, Rai, SK, Thakur, R, Chattopadhyay, P, Kar, SK. (2012). Bafibrinase: A non-toxic, non-hemorrhagic, direct-acting fibrinolytic serine protease from *Bacillus* sp. strain AS-S20-I exhibits in vivo anticoagulant activity and thrombolytic potency. *Biochimie*, 94:1300-1308.
- Narmatha, V, Bhavan, PS, Karthik, M, Srinivasan, V, Mahendran, R, Satgurunathan, T. (2017). *Lactobacillus fermentum* on ammonia reduction and growth promotion of *Macrobrachium rosenbergii* post larvae, and in vitro competitive exclusions of pathogenic bacteria. *Int. J. Fish. Aqua. Stud.*, 5(1):506–514.
- New, MB, Valenti, WC, Tidwell, JH, D'Abramo, LR, Kutty, MN. (2010). *Freshwater prawns: biology and farming*. Oxford: Wiley-Blackwell.
- Newman, MW, Lutz, PL, Snedaker, SC. (1982). Temperature effects and feed ingestion and assimilation of nutrients by the Malaysian prawn, *Macrobrachium rosenbergii* (de Man). *J. World Maricult. Soc.*, 13(1-4):95-103.
- Pinchuk, IV, Bressollier, P, Verneuil, B, Fenet, B, Sorokulova, IB, Megraud, F, Urdaci, MC. (2001). In Vitro Anti-*Helicobacter pylori* Activity of the Probiotic Strain *Bacillus subtilis* 3 Is Due to Secretion of Antibiotics. *Antimicrob. Agent. Chemotherapy*, 45(11):3156-3161.
- Raida, MK, Larsen, JL, Nielsen, ME, Buchmann, K. (2003). Enhanced resistance of rainbow trout, *Oncorhynchus mykiss* (Walbaum), against *Yersinia ruckeri* challenge following oral administration of *Bacillus subtilis* and *B. licheniformis* (BioPlus2B). *J. Fish Diseases*, 26:495–498.
- Ran, C, Carrias, A, Williams, MA, Capps, N, Dan, BCT, Newton, JC, Kloeppe, JW, Ooi, EL, Browdy, CL, Terhune, JS, Liles, MR. (2012). Identification of *Bacillus* Strains for Biological Control of Catfish Pathogens. *PLOS ONE*, 7(9):e45793.
- Rengpipat, S, Tunyanun, A, Fast, AW, Piyatiratitivorakul, S, Menasveta, P. (2003). Enhanced growth and resistance to *Vibrio* challenge in pond-reared black tiger shrimp *Penaeus monodon* fed a *Bacillus* probiotic. *Dis. Aquat. Org.*, 55:169-173.
- Roe, SH. (1955). The determination of sugar in blood and spinal fluid with anthrone reagent. *J. Biol. Chem.*, 212:334–343.

Saad, AS, Habashy, MM, Sharshar, KM. (2009). Growth Response of the freshwater prawn, *Macrobrachium rosenbergii* (De Man), to diets having different levels of Biogen®. *World Appl. Sci. J.*, 6:550–556.

Sambrook, J, Fritschi, EF, Maniatis, T. (1989). *Molecular cloning: a laboratory manual*, Cold Spring Harbor Laboratory Press, New York.

Seenivasan C, Radhakrishnan S, Shanthi R, Muralisankar T, Bhavan PS. (2014). Influence of probiotics on survival, growth, biochemical changes and energy utilization performance of *Macrobrachium rosenbergii* post-larvae. *Proceed. Zoolog. Soc.*, 68(1):74–83. DOI: 10.1007/s12595-014-0097-4.

Seenivasan, C, Bhavan, PS, Radhakrishnan, S, Muralisankar, T, Immanuel, G, Srinivasan, V, Manickam, N. (2013). Effect of *Saccharomyces cerevisiae* on survival, growth, biochemical constituents and energy utilization in the prawn *Macrobrachium rosenbergii*. *Int. J. Appl. Biol. Pharm. Technol.*, 4:39–47.

Seenivasan, C, Bhavan, PS, Radhakrishnan, S, Shanthi, R. (2012). Enrichment of *Artemia* nauplii with *Lactobacillus sporogenes* for enhancing the survival, growth and levels of biochemical constituents in the post larvae of the freshwater prawn *Macrobrachium rosenbergii*. *Turkish J. Fish. Aqua. Sci.*, 12:3–31.

Seenivasan, C, Bhavan, PS, Radhakrishnan, S. (2011). Effect of probiotics (Binifit™) on survival, growth, biochemical constituents and energy budget of the freshwater prawn *Macrobrachium rosenbergii* post larvae. *Elix. Aquacul.*, 41:5919–5927.

Sharma, S, Agarwal, N, Verma, P. (2012). Probiotics: The Emissaries of Health from Microbial World. *J. Appl. Pharm. Sci.*, 02(01):138-143.

Sudha, A, Bhavan, PS, Manjula, T, Kalpana, R, Karthik, M, (2019a). *Bacillus licheniformis* as a probiotic bacterium for culture of the prawn *Macrobrachium rosenbergii*. *Res. J. Life Sci. Bioinfor. Pharm. Chem. Sci.*, 5(4):44-61.

Sudha, A, Bhavan, PS, Udayasurian, R, Manjula, T, Kalpana, R, Karthik, M. (2019b). Molecular Identification of Gut Microflora of the Prawn *Macrobrachium rosenbergii* Fed with Probiotic Bacterium *Bacillus licheniformis* Supplemented Diet. *Haya Saudi J. Life Sci.*, 4(9):303-317.

Sumi, H, Nakajima, N, Yatagai, C. (1995). A unique strong fibrinolytic enzyme (Katsuwokinase) in *Skipjack shiokara*, a Japanese traditional fermented food, *Comp. Biochem. Physiol.* 112B: 543-547.

Suralikar, V, Sahu, NP. (2001). Effect of Feeding Probiotic (*Lactobacillus cremoris*) on Growth and Survival of *Macrobrachium rosenbergii* Post Larvae. *J. Appl. Anim. Res.*, 20(1):17–124.

Tekinay, AA, Davies, SJ. (2001). Dietary carbohydrate level influencing feed intake, nutrient utilisation and plasma glucose concentration in the rainbow trout, *Oncorhynchus mykiss*. *Turkish J. Vet. Animal Sci.*, 25:657–666.

Teshima, S, Koshio, S, Kanazawa, A, Oshida, K. (1992). Essential fatty acids of the prawn *Macrobrachium rosenbergii*. In: Abstracts of the 3rd Asian Fisheries Forum, 26-30 October, Singapore, p. 90. Asian Fisheries Society, Manila, Philippines.

Urdaci, MC, Pinchuk, I. (2004). Antimicrobial Activity of *Bacillus* Probiotics. Book Chapter-15, In: Bacterial spore formers – Probiotics and emerging applications (Edition: Ed. Horizon Bioscience, Norfolk, U.K).

Vaseeharan, B, Ramasamy, P. (2003). Control of pathogenic *Vibrio* spp. by *Bacillus subtilis* BT23, a possible probiotic treatment for black tiger shrimp *Penaeus monodon*. *Lett. Appl. Microbiol.*, 36:83-87.

Venkat, HK, Sahu, NP, Jain, KK. (2004). Effect of feeding *Lactobacillus* based probiotics on the gut microflora, growth and survival of post larvae of *Macrobrachium rosenbergii* (de Man). *Aquacul. Res.*, 35:501–507.

Venkataramani, VK, Rajagopalasamy, CBT, Ravi, D. (2002). Effect of formulated feeds on the growth and broodstock development in *Macrobrachium rosenbergii*. *Asian Fish. Sci.*, 15: 357-364.

Wang, SL, Wu, YY, Liang, TW. (2011). Purification and biochemical characterization of a nattokinase by conversion of shrimp shell with *Bacillus subtilis* TKU007, *New Biotech.*, 28(2):196-202.

How to cite this article: Periakali Saravana Bhavan, Duraisamy Anitha, Thankaraj Manjula, Rajendran Udayasuriyan, Ramasamy Kalpana, Madhayan Karthik, Survival and Growth Promoting Effects of *Bacillus Subtilis* on *Macrobrachium Rosenbergi*, and Validation of Gut Microflora. *International Journal of Advanced Biological and Biomedical Research*, 2020, 8(4), 440-460. Link: http://www.ijabbr.com/article_43288.html