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Research Article

Growth Promoting Potential and Colonization Ability of Probiotics (*Bacillus coagulans* and *Bacillus subtilis*) on the Freshwater Prawn *Macrobrachium rosenbergii* Post-Larvae

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Abstract

The probiotic effects of *Bacillus coagulans* and *Bacillus subtilis* were studied on survival, growth, concentrations of basic biochemical constituents, activities of digestive enzymes, and their colony establishments in the gut of *Macrobrachium rosenbergii* post-larvae (PL). Eleven groups of PL (2.03±0.05 in length and 0.18±0.01g in weight), each consists of 35 individuals maintained in 25 L of ground water and fed *ad libitum* with five serially diluted concentrations, 10^{-1}, 10^{-3}, 10^{-5}, 10^{-7} and 10^{-9} of *B. coagulans*, and *B. subtilis* incorporated diets containing 40% protein, for 45 days. Diet without incorporation of any of these probiotics was served as control. These probiotics were found to be alive in the respective feed even on day-15 after their formulations. Significant improvement in survival, nutritional indices (weight gain, specific growth rate, food conversion ratio and protein efficiency ratio), contents of basic biochemical constituents (total protein, amino acid, carbohydrate and lipid) and activities of digestive enzymes (protease, amylase and lipase) were observed ($P<0.05$), particularly in 10^{-7} concentration of *B. coagulans*, and *B. subtilis* incorporated diets fed PL when compared with control. The biochemical confirmation tests revealed that presence of *Escherichia coli*, *Acetonobacter* sp., *Salmonella* sp., and *Pseudomonas* sp., in the gut of control PL. In the gut of PL fed with *B. coagulans* incorporated diet, *Acetonobacter* sp., *Salmonella* sp., and *Pseudomonas* sp., were found to be competitively excluded, whereas, in the gut of PL fed with *B. subtilis* incorporated diet, *Acetonobacter* sp., and *Salmonella* sp., only were found to be excluded competitively. Actually, colonies of *Bacillus* sp., and *Lactobacillus* sp., were found to be establishment in the gut of PL fed with *B. coagulans*, and *B. subtilis* incorporated diets. Overall, these probiotics incorporated diets produced better growth and survival due to better FCR and activities of digestive enzymes, which in turn led to better nutritional profile. Therefore they are recommended as feed additives for sustainable culture of *M. rosenbergii*.

Introduction

Aquaculture is one of the fastest growing food sectors in the world with main a Moto to supply rich protein food to the growing human population [1]. Beyond the fishes and other animals, the crustaceans, such as prawns, shrimps, crabs, lobsters and crayfish have a vital role in augmentation of protein production [2]. The freshwater prawn, *Macrobrachium rosenbergii* considered to be one of the crustacean species with increasing potential for aquaculture because of its commercial value and nutritious delicacy for human consumption cells added as dietary supplements to improve the health of aquatic animals. Generally, 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 and stimulating host immunity [3]. Studies on *M. rosenbergii* with some probiotics, *Lactobacillus sporogenes*, *Bacillus subtilis, Saccharomyces cerevisiae, Lactobacillus acidophilus, Lactobacillus rhamnosus, Bifidobacterium longum, Bifidobacterium bifidum, Saccharomyces boulardii*, *Clostridium butyricum, Bacillus coagulans* and *Lactobacillus brevis,* and their products,

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Biogen, Binifit™, LactoBacil®$_{plus}$ and ViBact* [4-14], showed enhanced growth, survival, and nutritional profiles due to improved general health by establishing their colonies in the gut. *Bacillus coagulans* is a Gram-positive, lactic acid forming bacterial species. It is a good candidate for probiotic use, produces organic acids and possesses the capacity to sporulate. It secretes a bacteriocin, coagulin, which has activity against a broad spectrum of enteric microbes [15-17]. *Bacillus subtilis* is also a Gram-positive, lactic acid-forming bacterial species. A strain of *B. subtilis* 2335 has the ability to produce the antibiotic, Amicoumacin, which showed an *in-vitro* activity against *Helicobacter pylori* [18].

In the present study, *B. coagulans* and *B. subtilis* was individually incorporated at different concentrations with formulated diets and fed to *M. rosenbergii* PL for assessing their ability on promotion of growth and survival by enhancing the contents of basic biochemical constituents (total protein, amino acid, carbohydrate and lipid), and activities of digestive enzymes (protease, amylase and lipase). This was further to recommend the aquaculture industry with their optimum concentrations. Furthermore, to evaluate their competitive exclusion abilities of pathogenic bacteria, their colony establishments in the gut of PL were confirmed biochemically.

Materials and Methods

Procurement of *Macrobrachium rosenbergii* PL and acclimation

The post larvae (PL-20) of *M. rosenbergii* were procured from the Nursery pond at Singanallur, Coimbatore, Tamil Nadu, India. They were transported to the laboratory in polythene bags half filled with oxygenated pond water. They were then acclimated to the ambient laboratory condition in cement tanks (6×3×3 feet) filled with ground-water for 2 weeks. The ground water satisfied the required physico-chemical parameters (Table 1).

During acclimation the prawns were fed with boiled egg albumin, live *Artemia* nauplii, and commercially available scampi feed. About 50% of tank water was routinely renewed every day to maintain a healthy environment. Aeration was also provided. These ensured sufficient oxygen supply to the prawns and an environment devoid of accumulated metabolic wastes. The unfed feeds, feces, molt and dead prawns were removed by siphoning.

Procurement of probiotics and their sub-culture

The lyophilized powder of *Bacillus coagulans* (MTCC 2302) and *Bacillus subtilis* (MTCC 121) were procured from Microbial Type Culture Collection (MTCC), Chandigarh, India. It was subjected to sub-culture with nutrient broth (Hi-media, India) containing the following ingredients as per manufacturer's protocol (Table 2) and incubated at 37 °C for 12 hours to observe their growth.

Agar (12.0 g L^{-1}) was added with the nutrient broth, and the agar plates were incubated for 24 h at 37°C for checking the sterility. Then 20 µL broth culture of *B. coagulans*, and *B. subtilis* were separately spread over the agar media (nutrient+agar) and kept for 24 h at 37 °C to observe their growth (Figure 1).

Table 1: The physico-chemical parameters of the ground water used in the laboratory.

Parameter	Device/ Methodology	Value
Temperature (°C)	Mercury thermometer (Jenson & Nicholson (India) Limited, Kolkata)	22±0.2
pH	ESICO, India, µP Based Water and Soil Analysis Kit, Model 1160	7.1±0.20
TDS (g/l)	APHA method (2005) [19]	0.96±0.07
DO_2 (mg/l)	Winkler's method (1888) [20]	6.10±0.30
Salinity (mg/l)	ESICO, India, µP Based Water and Soil Analysis Kit Model 1160	0.63±0.01
EC (mS/cm)	ESICO, India, µP Based Water and Soil Analysis Kit, Model 1160	1.01±0.01
Ammonia (mg/l)	Phenol hypochloride method of Solorzano (1969) [21]	0.030±0.007

TDS, total dissolved solids; DO, dissolved oxygen; EC, Electrical conductivity.

Table 2: Nutrient broth for *B. coagulans* and *B. subtilis* sub-culture.

Ingredient compositions	Quantity (g L^{-1})
Peptic digest of animal tissue	5.0
Sodium chloride	5.0
Beef extract	1.5
Yeast extract	1.5
Final pH (at 25°C)	7.4

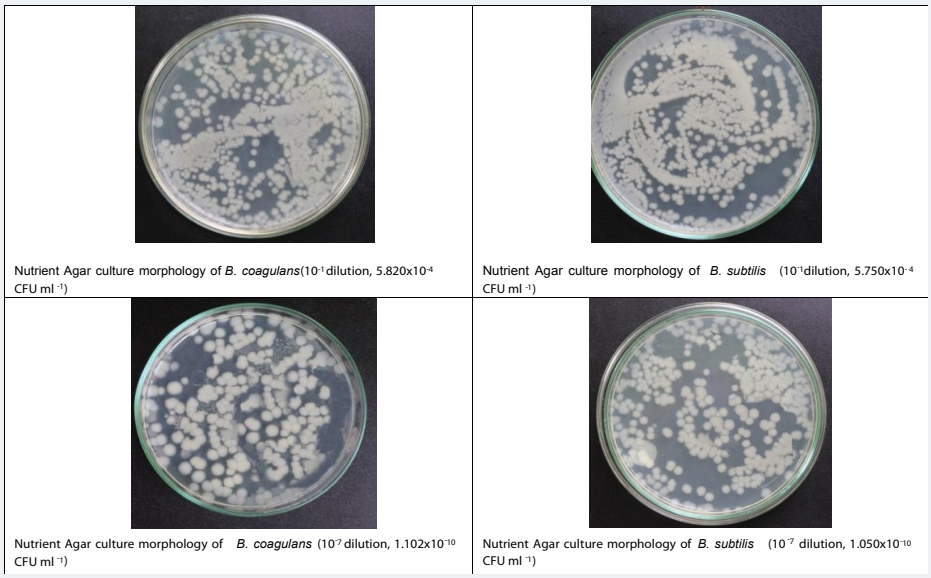


Figure 1: Nutrient Agar culture morphology of *B. coagulans*, and *B. subtilis*.

Feed preparation

The branded feed ingredients, included fishmeal, soybean meal, groundnut oilcake, wheat bran, tapioca flour, cod liver oil and egg were purchased from a local market. A Vitamin-B complex with vitamin-C purchased from a local pharmacy was also added. The experimental diets were prepared with selected feed ingredients as per "Pearson's square-method" using pre determined value of 45% protein content. Fishmeal, soybean meal and groundnut oilcake were used as protein sources; wheat bran and tapioca flour were used as carbohydrate sources; sunflower oil was used as lipid source; tapioca flour and egg albumin were served as binding agents; vitamin B complex with vitamin C was also added as essential micronutrients (Table 3).

The proportion of each ingredient required was calculated precisely for the premix along with tapioca powder, stream cooked and cooled at room temperature (28ºC). Egg albumin, sunflower oil, and vitamin B-complex with vitamin-C were then added one by one. *B. coagulans*, and *B. subtilis* were separately incorporated into the basal diet at 10^{-1}, 10^{-3}, 10^{-5}, 10^{-7} and 10^{-9} concentrations, each (diet-1 to diet-10). Diet '0' (without incorporation of any probiotic served as control. The dough was prepared for each formulation and pelletized separately to 2 mm pellets. These were dried in a thermostatic oven (M/s. Modern Industrial, Mumbai, India) at 40ºC until they reached constant weight, and stored in airtight jars at room temperature (28ºC). The proximate composition of organic matters was determined according to AOAC [22]. The feeds were then placed in a hot air oven at slightly >100ºC and the loss of weight determined as the moisture content. The concentrations of minerals were estimated by using atomic absorption spectrophotometer (AAS) method (Table 4).

Viability of probiotics in the respective diets

The diet was freshly prepared once every 15 days to ensure high probiotic viability throughout the feeding trail. One gram of each diet prepared with10^{-7} diluted probiotic

concentration was taken and dissolved in autoclaved double distilled water (10 mL). A volume of 20µL was spread over MRS agar medium, incubated at 37ºC for 24 h and the colony morphology observed was compared with the original *B. coagulans*, and *B. subtilis* sub-cultures morphology. The growth was recorded in all the culture plates except control. Therefore, the incorporated *B. coagulans*, and *B. subtilis* were alive in their respective diets even on day-15 after their formulation (Figure 2).

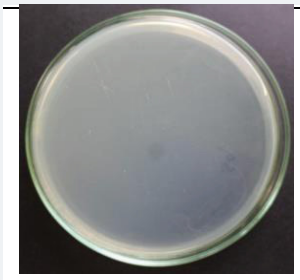
Table 3: Ingredients used to formulate the basal diet.

Basal ingredients (BI)	g/ 100 g
Fish meal	25
Groundnut oil cake	25
Soybean meal	25
Wheat bran	10
Egg albumin	7
Tapioca flour	5
Sunflower oil	2
Vitamin mix*	1
Total	100

*BECOSULES CAPSULES, manufactured by Pfizer. Each capsule contains: Thiamine mononitrate (IP)-10 mg; Riboflavin (IP)-10 mg; Pyridoxine hydrochloride (IP)-3 mg; Vitamin B12 (as tablets 1:100) (IP)-15 mcg; Niacinamide (IP)-100 mg; Calcium pantothenate (IP)-50 mg; Folic acid (IP)-1.5 mg; Biotin (USP)-100 mcg; Ascorbic acid (IP)-150 mg.

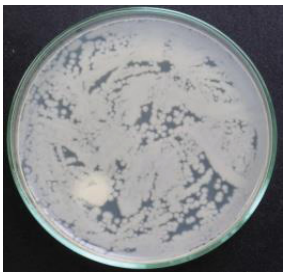
Table 4: Proximate components of basal diet prepared using various basal ingredients.

Proximate components	Quantity (%)
Crude protein	45.88
Total Nitrogen-free extract (carbohydrate)	33.55
Ether extract (crude fat)	7.28
Crude fiber	1.57
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	175.87 ppm
Copper	25.86 ppm
Salt	0.56



Control diet shows no growth of any bacterium

Experimental feed incorporated with *B.coagulans* (10 1.102x1 0^{-10} CFU ml^{-1}) shows its presence and growth $^{-7}$ dilution,



Experimental feed incorporated with *B.subtilis* (10^{-7} dilution, 1.050x10^{10} CFU ml^{-1}) shows its presence and growth

Figure 2: Viability of *B. coagulans*, and *B. subtilis* in experimental diets on day-15 after they were prepared.

Feeding trial

M. rosenbergii PL-35 (2.03±0.05 in length and 0.18±0.01g in weight) were acclimated in plastic aquaria for three days during which they starved for 24 hr before the commencement of the experiment. The feeding trail lasted 45 days. Four groups of 35 prawns were placed in 25 L aquaria in triplicate groups. The experimental groups were fed with the respective concentrations of *B. coagulans*, and *B. subtilis* incorporated feeds twice a day (8:00 AM and 8:00 PM) at 10% of body weight. All the water was renewed daily and aerated constantly. The uneaten food particles, faeces, moults and dead prawns were removed by siphoning.

Analysis of nutritional indices

On the final day of feeding trial the morphometric data, such as the final length and weight were measured for calculating the growth parameters, such as survival rate (SR), Length gain (LG), weight gain (WG), specific growth rate (SGR), food conversion ratio (FCR), food conversion efficiency (FCE) and protein efficiency ratio (PER) [23].

SR (%)=Total No. of PL alive at the end of experiment/ Total No. of PL introduced at the beginning of the experiment×100.

LG (cm)=(Final length (cm)-initial length (cm). Weight gain, WG(g)=Final weight(g)-initial weight (g).

SGR(%)=*log* of final weight (g)-*log* of initial weight (g)/ No. of experimental days×100.

FCR(g)=Food consumed (g)/ WG (g).

PER(g)=WG (g)/protein intake (g).

Estimation of concentrations of biochemical constituents

On the final day, the concentrations of basic biochemical constituents were determined. Concentration of total protein was estimated by the method of [24]. The concentration of total amino acid was analyzed by according to Moore and Stein [25]. The concentration of total carbohydrate was estimated by the method of Roe [26]. The concentration of total lipid was determined according to [27] and estimated by the method of [28]. For these parameters, tissues from five prawns were pooled together from each group to constitute a single observation and three such observations were made to fulfill the triplicate analysis.

Assays of digestive enzymes activities

Activities of digestive enzymes were assayed before and after the feeding trial. The digestive tract of three prawns from each replicate were carefully dissected and homogenized in ice-cold distilled water and centrifuged at 9300 g under 4ºC for 20 min. The supernatant was used as a crude enzyme source. Total protease activity was determined by casein-hydrolysis method of Furne et al. [29], where one unit of enzyme activity represented the amount of enzyme required to liberate 1µg of tyrosine per minute. Amylase activity was determined according to Bernfeld et al. [30], and the specific activity of amylase was calculated as milligrams of maltose liberated per gram of protein per hour (mg/g/h). Lipase activity was determined by the method of Furne et al. [29]. One unit of lipase activity was defined as the amount of free fatty acid released from triacylglycerol per unit time estimated by the amount of NaOH required to maintain pH constant and represented as milli equivalents of alkali consumed.

Analysis of gut microbial colonization

The gut of control PLs, and the gut of experimental PLs fed with the best concentration of *B. coagulans*, and *B. subtilis* supplemented diet (10^{-7} dilution of each

probiotic) were subjected to bacterial analysis. The PLs were deactivated by kept them in freezer at -20ºC for 10 minutes. The PL surface was sterilized with 50 ppm formalin for 30 seconds to remove the external flora. The digestive tract was dissected and homogenized with phosphate buffered saline (pH-7.2) under aseptic condition. The homogenate was serially diluted up to 10^{-4} dilution. A volume of 0.5 mL of aliquot was mixed with agar nutrient broth and cultured for 24 h at 35ºC. Then, 0.1 mL broth culture was seeded over the surface of freshly prepared nutrient agar plates and incubated at 37 ºC for 24 h. The appearance of different bacterial colony was identified and confirmed through routine bacteriological tests [31]. The bacterial colonies were enumerated [Bacteria count (CFU/g)=Number of colonies×Dilution factor/ Volume of sample (g)].

Statistical Analysis

One way analysis of variance (ANOVA) using SPSS package (version 20.0) was used to determine the variations between control and treatment values, and between treatments, followed by Duncan multiple range test (DMRT) and the significances at $P<0.05$ are mentioned. The data are represented as means±standard deviations.

Results and Discussion

Growth and nutritional indices

The survival, growth (LG and WG), specific growth rate, protein efficiency ratio were significantly increased in the prawns fed with *B. coagulans*, and *B. subtilis* supplemented diets when compared with control ($P<0.05$). Among the different concentrations, 10^{-7} dilution of each probiotic incorporation was produced the best performance on these parameters. The lowest FCR recorded in this concentration of *B. coagulans*, and *B. subtilis* incorporations reflected the superior quality of the diets formulated (Tables 5 and 6).

Table 5: Nutritional indices of *M. rosenbergii* PL fed with *B. coagulans* incorporated feeds (initial length and weight of the PL: 2.86 cm; 0.39 g).

Parameter	Control	Concentrations of *B. coagulans*					F-value
		10^{-1}	10^{-3}	10^{-5}	10^{-7}	10^{-9}	
SR (%)	87.61±1.64^{e}	89.52±1.64^{d}	91.42±2.85^{bc}	93.33±1.64^{ab}	95.23±1.64^{a}	92.38±2.85^{abc}	6.12
Length(cm)	3.73±0.05^{f}	4.03±0.05^{e}	4.26±0.10^{d}	4.66±0.11^{b}	4.98±0.01^{a}	4.44±0.05^{c}	146.68
LG(g)	0.86±0.05^{f}	1.16±0.04^{e}	1.40±0.05^{d}	1.80±0.10^{b}	2.11±0.07^{a}	1.57±0.04^{c}	105.14
Weight(cm)	0.78±0.03^{f}	0.93±0.01^{e}	1.14±0.01^{d}	1.45±0.04^{b}	1.61±0.01^{a}	1.33±0.01^{c}	476.46
WG (g)	0.39±0.04^{f}	0.54±0.02^{e}	0.75±0.02^{d}	1.06±0.04^{b}	1.22±0.02^{a}	0.94±0.02^{c}	340.76
SGR (%)	0.76±0.03^{e}	0.87±0.02^{d}	0.96±0.02^{c}	1.07±0.02^{ab}	1.11±0.02^{a}	1.03±0.02^{b}	63.61
FCR (g)	3.53±0.14^{a}	3.24±0.12^{b}	2.49±0.06^{c}	2.12±0.08^{d}	1.69±0.02^{e}	2.00±0.04^{d}	169.41
PER (g)	0.33±0.03^{e}	0.76±0.07^{d}	0.94±0.02^{c}	1.10±0.04^{b}	1.38±0.23^{a}	1.17±0.02^{b}	229.80

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, SGR, Specific Growth Rate; FCR, Food Conversion Ratio; PER, Protein Efficiency Ratio.

Table 6: Nutritional indices of *M. rosenbergii* PL fed with *B. subtilis* incorporated feeds (initial length and weight of the PL: 2.86 cm; 0.39 g).

Parameter	Control	Concentrations of *B. subtilis*					F-value
		10^{-1}	10^{-3}	10^{-5}	10^{-7}	10^{-9}	
SR (%)	87.61±1.64^{c}	90.46±1.64^{bc}	91.42±2.85^{bc}	94.28±2.85^{ab}	96.19±1.64^{a}	93.33±1.64^{ab}	6.10
Length(cm)	3.33±0.05^{f}	3.53±0.05^{e}	3.96±0.05^{d}	4.40±0.10^{b}	4.73±0.05^{a}	4.26±0.05^{c}	192.12
LG(g)	0.46±0.05^{e}	0.66±0.05^{d}	1.23±0.15^{c}	1.53±0.05^{b}	1.86±0.05^{a}	1.40±0.10	109.44
Weight(cm)	0.78±0.03^{f}	1.03±0.01^{e}	1.20±0.02^{d}	1.46±0.01^{b}	1.78±0.01^{a}	1.38±0.02^{c}	713.39
WG (g)	0.39±0.04^{f}	0.63±0.02^{e}	0.82±0.01^{d}	1.05±0.03^{b}	1.39±0.02^{a}	0.99±0.03^{c}	525.05
SGR (%)	0.80±0.03^{e}	0.91±0.03^{d}	0.99±0.01^{c}	1.11±0.02^{a}	1.16±0.02^{a}	1.04±0.02^{b}	69.36
FCR (g)	4.11±0.43^{a}	2.66±0.10^{b}	2.17±0.03^{c}	1.65±0.01^{de}	1.46±0.02^{e}	1.85±0.05^{c}	83.98
PER (g)	0.15±0.01^{f}	0.88±0.03^{e}	1.08±0.01^{d}	1.41±0.01^{b}	1.69±0.02^{a}	0.97±0.03^{c}	1.81

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, SGR, Specific Growth Rate; FCR, Food Conversion Ratio; PER, Protein Efficiency Ratio.

Concentrations of basic biochemical constituents and activities of digestive enzymes

The concentrations of total protein, amino acid, carbohydrate and lipid were significantly increased ($P<0.05$) in test prawns fed with *B. coagulans*, and *B. subtilis* supplemented diets when compared with control (Tables 7 and 8). These were because of the significant increases ($P<0.05$) in activities of protease, amylase and lipase (Tables 7 and 8), which ultimately produced better digestion and absorption of nutrients. Among the different concentrations, 10^{-7} dilution of each probiotic incorporation was produced the best performance on these parameters, which in turn responsible for better survival and growth of *M. rosenbergii.*

Gut microbial population

The biochemical confirmation tests revealed that presence of *Escherichia coli*, *Acetonobacter* sp., *Salmonella* sp., and *Pseudomonas* sp., in the gut of control PL. In the gut of PL fed with *B. coagulans* incorporated diet, *Acetonobacter* sp., *Salmonella* sp., and *Pseudomonas* sp., were found to be competitively excluded, whereas, in the gut of PL fed with *B. subtilis* incorporated diet, *Acetonobacter* sp., and *Salmonella* sp., only were found to be excluded competitively. Actually, colonies of *Bacillus* sp., and *Lactobacillus* sp., were found to be establishment in the gut of PL fed with *B. coagulans*, and *B. subtilis* incorporated diets (Tables 9,10; Figsures 3-5).

Table 7: Concentrations of basic biochemical constituents and activities of digestive enzymes in *M. rosenbergii* PL fed with *B. coagulans* incorporated feeds.

Parameters		Control	Concentrations of *B. coagulans*					F-value
			10^{-1}	10^{-3}	10^{-5}	10^{-7}	10^{-9}	
Biochemical (mg/g wet. tissue)	Protein	42.70±1.70^{e}	50.53±2.27^{d}	58.18±2.00^{c}	71.2±1.22^{b}	80.83±2.36^{a}	62.95±1.95^{c}	121.90
	Amino acid	20.97±1.20^{f}	29.17±0.80^{e}	37.05±1.50^{d}	48.63±1.78^{b}	54.39±1.15^{a}	42.37±1.56^{c}	131.96
	Carbohydrate	12.58±0.30^{f}	14.19±0.50^{e}	18.19±0.82^{d}	24.82±0.78^{b}	27.97±0.91^{a}	21.24±0.52^{c}	235.47
	Lipid	6.96±0.21^{f}	10.24±0.58^{e}	14.84±0.15^{d}	18.38±0.39^{b}	23.96±0.41^{a}	17.6±0.31^{c}	688.12
Digestive Enzymes (U/ mg protein)	Protease	1.86±0.13^{e}	2.33±0.12^{d}	2.74±0.11^{c}	2.99±0.11^{c}	3.27±0.10^{a}	2.80±0.01^{bc}	56.68
	Amylase	0.73±0.09^{e}	1.09±0.09^{d}	1.38±0.14^{d}	2.07±0.08^{b}	2.31±0.08^{a}	1.81±0.08^{c}	110.65
	Lipase*	0.20±0.01^{f}	0.43±0.01^{e}	0.50±0.01^{d}	0.57±0.01^{b}	0.62±0.07^{a}	0.54±0.01^{c}	489.45

Each value is mean ± standard deviation of three individual observations. *, unit×10^{3}
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).

Table 8: Concentrations of basic biochemical constituents and activities of digestive enzymes in *M. rosenbergii* PL fed with *B. subtilis* incorporated feeds.

Parameters		Control	Concentrations of *B. subtilis*					F-value
			10^{-1}	10^{-3}	10^{-5}	10^{-7}	10^{-9}	
Biochemical (mg/g wet. tissue)	Protein	46.68±3.21^{f}	55.67±2.45^{e}	62.10±3.34^{d}	76.01±1.85^{b}	86.18±2.45^{a}	68.51±2.45^{c}	84.86
	Amino acid	22.62±2.82^{f}	39.60±2.00^{e}	41.47±2.15^{d}	51.37±2.15^{b}	60.80±1.41^{a}	46.66±1.41^{c}	132.25
	Carbohydrate	12.65±0.74^{a}	15.13±1.55^{a}	20.54±1.77^{a}	24.81±1.01^{a}	29.60±1.31^{a}	22.03±0.46^{a}	1.00
	Lipid	8.32±0.47^{f}	11.09±0.57^{e}	16.87±1.54^{d}	20.17±0.34^{b}	24.28±0.60^{a}	20.90±0.74^{c}	925.15
Digestive Enzymes (U/ mg protein)	Protease	1.60±0.13^{d}	1.80±0.18^{c}	2.01±0.06^{b}	2.17±0.06^{b}	2.64±0.10^{a}	2.03±0.05^{b}	29.96
	Amylase	0.42±0.05^{e}	0.73±0.09^{e}	1.13±0.09^{d}	1.49±0.47^{bc}	2.14±0.08^{a}	1.64±0.04^{b}	25.67
	Lipase*	0.18±0.01^{e}	0.39±0.01^{d}	0.41±0.02^{cd}	0.48±0.02^{b}	0.55±0.02^{a}	0.43±0.01^{bc}	138.04

Each value is mean ± standard deviation of three individual observations.*, unit×10^{3}
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).

Table 9: Colony formation units (CFU) of *B. coagulans* and *B. subtilis* in the gut of *M. rosenbergii* PL fed with respective probiotics incorporated diets at their best concentrations.

Isolated species	Control	B. coagulans	B. subtilis
E. coli	P (4.096×10^{-7})	P (2.123×10^{-7})	P (3.425×10^{-7})
Acetonobacter sp.	P (3.250×10^{-7})	A	A
Salmonella sp.	P (3.252×10^{-7})	A	A
Pseudomonas sp.	P (4.420×10^{-7})	A	P (2.845×10^{-7})
Bacillus sp.	A	P(3.645×10^{-7})	P (3.128×10^{-7})
Lactobacillus sp.	A	P(3.515×10^{-7})	P (7.038×10^{-7})

P, Present; A, Absent; **CFU**, Colony Formation Unit (originally counted at 10^{-4}).

Table 10: Confirmative results of biochemical tests for microflora in the gut of *M. rosenbergii* PL fed with *B. coagulans*, and *B. subtilis* incorporated diets.

Test	Control				*B. coagulans*			*B. subtilis*			
	E.c	Ac.	Sa.	Ps.	E.c	Ba.	La.	E.c	Ps.	Ba.	La.
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	-	-	-	+	-	+	+	-	+	+	+
Glucose test	A	A	A	A	A	A	A	A	A	A	A
mLactose test	A	A	A	NA	A	A	A	A	NA	A	A
Sucrose test	A	A	A	A	A	A	A	A	A	A	A
Manitol test	A	A	A	A	A	NA	A	A	A	NA	A
Maltose test	Na	NA	NA	A	Na	NA	NA	Na	A	NA	NA

+, Positive; **-**, Negative; **A**, Acid production; **NA**, No acid production; **E.c**, *E. coli*; **Ac**, *Acinetobacter* sp.; **Sa**, *Salmonella* sp.; **Ps**, *Pseudomonas* sp.; **Ba**, *Bacillus* sp.; **Ls**, *Lactobacillus* sp.

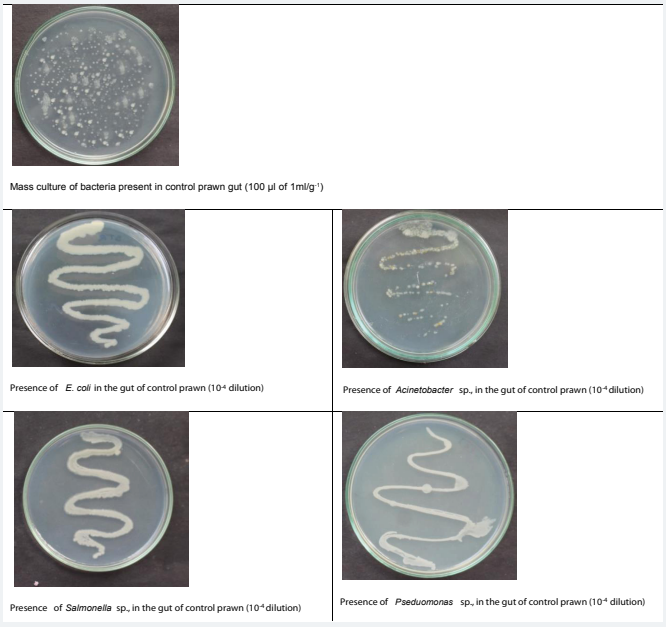


Figure 3: Nutrient agar streak plate morphology of bacteria present in control prawn gut.

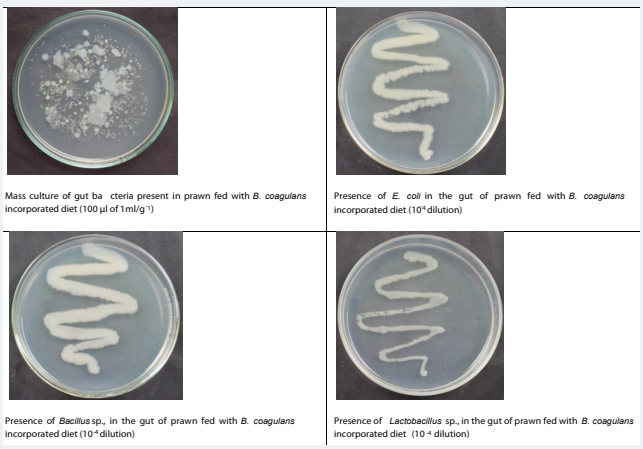


Figure 4: Nutrient agar streak plate morphology of bacteria present in the gut of prawns fed with *B. coagulans* (10^{-7} dilution, $1.102x10^{-10}$ CFU ml^{-1}) incorporated diet.

Figure 5: Nutrient agar streak plate morphology of bacteria present in the gut of prawns fed with *B. subtilis* (10^{-7} dilution, $1.050x10^{-10}$ CFU ml^{-1}) incorporated diet..

These results indicated that the supplemented probiotics have the characteristics ability to enhance the digestion, absorption of nutrients and alteration in the intestinal microflora, which led to better growth and survival of *M. rosenbergii*. It has been reported that the SR, WG, SGR, FCR and PER, carcase proximate composition, and activities of protease, amylase and lipase were significantly improved in *M. rosenbergii* fed with different probiotics, *L. sporogenes*, *L. rhamnosus, L. ceremoris*, *L. acidophilus, B. subtilis*, *C. butyricum*, *B. coagulans*, *S. cerevisiae* and commercial probiotic products, LactoBacil®$_{plus}$, ViBact*, Binifit™ and Biogen® incorporated diets [4-13,32-42]. Similarly, the dietary administration of *Lactobacillus plantarum*, *Bacillus* sp., and *C. butyricum* also produced a significant improvement in growth, carcase biochemical constituents, and activities of digestive enzymes in shrimps, *Litopenaeus vannamei* and *Marsupenaeus japonicas* [12,43-46].

It has been reported that *Lactobacillus* sp., supplemented feed significantly decrease the pathogenic bacteria (Enterobacteriacea, *Aeromonas* sp., and *Pseudomonas* sp.) in *M. rosenbergii* [47]. The probiotics play an important role in healthy maintenance gut microflora of the host, which prevents colonization of harmful bacteria and stimulates the immune system [48-51]. The presence of at least 10 bacterial genera *Escherichia coli, Proteus* sp., *Lactococcus* sp., *Enterobacteria sp., Lactobacillus* sp., *Pseudomonas* sp., *Staphylococcus* sp., *Bacillus* sp., *Klebsiella* sp., and *Streptococcus* sp., have been isolated from the gut of fishes *Oreochromis niloticus* and *Clarias gariepinus* [52].

Conclusion

Overall, these probiotics incorporated diets produced better growth and survival due to better FCR and activities of digestive enzymes, which in turn led to better nutritional profile. *B. coagulans*, and *B. subtilis* incorporated diets possessed the ability of competitively exclude *Acetonobacter* sp., *Salmonella* sp., and *Pseudomonas* sp., and *Acetonobacter* sp., and *Salmonella* sp., respectively. Therefore they are recommended as feed additives for sustainable culture of *M. rosenbergii*.

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