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# *BACILLUS LICHENIFORMIS* AS A PROBIOTIC BACTERIUM FOR CULTURE OF THE PRAWN *MACROBRACHIUM ROSENBERGII*

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**ABSTRACT:** This work emphasized the growth performance of the prawn, *Macrobrachium rosenbergii* post larvae (PL) supplemented with a probiotic bacterium, *Bacillus licheniformis* at five different serially diluted concentrations ($10^{-2}$, $10^{-4}$, $10^{-6}$, $10^{-8}$ and $10^{-10}$) respectively. After 90 days of feeding trial, in $10^{-6}$ (CFU, $935x10^{-6}$) dilution supplemented feed fed prawn produced significantly ($P<0.05$) increased survival rate, growth rate, contents of total protein, amino acid, carbohydrate, lipid and digestive enzymes activities (protease, amylase and lipase)when compared with control. The gut microfloral study showed the presence of *Pseudomonas* sp., *Klebsiella*., *Escherichia coli*., *Bacillus* sp., *Streptococcus* sp., *Staphylococcus* sp., *Citrobacter* sp., and *Acinitobacter* sp., in control prawn. The gut of experimental PL fed with *B. Licheniformis* showed presence of *Bacillus* sp., *Lactobacillus* sp., *E. coli*., *Streptococcus* sp., and *Citrobacter* sp. Thus, the pathogenic bacteria, such as *Pesudomonas* sp., *Klebsiella* sp., *Staphylococcus* sp., and *Acinitobacter* sp., were found to be competitively excluded in *B. licheniformis* supplemented feed fed prawn's gut.

**KEYWORDS:** Probiotics, Prawn, *B. licheniformis*, Pathogenic bacteria.

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## 1. INTRODUCTION

The aquaculture sector plays a crucial role as one of the potential sources of nutritional security and food safety. Many aquatic animals, like fishes, prawns, shrimps, crabs, lobsters, crayfish, holothurians, clams, chanks, green frog etc., are a rich source of protein, fatty acids, amino acids, antioxidants, vitamins and minerals and serve as a healthy choice for human consumption.

Presently, the country ranks third in the world in total fish production with an annual fish production of about 4.9 MMT. The world production of marine and freshwater crustaceans contributed 17.3% and 4.5%, respectively during 2014. The freshwater prawn, *Macrobrachium rosenbergii* has gained increased interest in recent years, due to its tolerance in wide variety of environmental conditions, presence of good level of essential nutrients (particularly protein), delicious taste and high economic value [1]. It has rich demand in both domestic and international markets, and thus earns very good foreign exchange. It requires 30 to 45% crude protein and good culture environment for good growth and yield [2]. It generates employment opportunity. Probiotics may provide fatty acids, vitamins and essential nutrients, which play a vital role in improving the growth, stress tolerance and disease resistance. Gatesoupe [3] defined probiotics as living microbial cells added as dietary supplements, which improve the health of hosts. Probiotic treatments may provide a broader spectrum and greater non-specific disease protection through competitive exclusion and immune modulation, and such non-invasive therapies restore the natural gut flora [4, 5]. Crustacean possesses a non-specific immune response. According to Elsersy and Mohamed [6], some *B. licheniformis* strains can produce exotoxins with strong hemolytic activity, causing a reduction in aquaculture products. *B. licheniformis* was never used as probiotic in the shrimp culture. However, use of commercial *Bacillus subtilis* and *B. licheniformis* as probiotics either in a single form or in the combination manner has raised a promising hope for aquaculture species including shrimp [7]. Seenivasan *et al*., [8-15] Jayanthi *et al*., [16, 17] Karthik *et al*., [18, 19] and Manjula *et al*., [20] have extensively reported that commercial probiotic products (Binifit$^{TM}$, LactoBacil$^{®plus}$ and ViBact*), individual and combined probiotics (*Lactobacillus sporogenes, B. subtilis*, *Lactobacillus brevis* and *Bacillus coagulans*) have promoted the survival, growth, and total protein, carbohydrate and lipid, and profiles of amino acids and fatty acids, and activities of digestive enzymes (protease, amylase and lipase) in *M. rosenbergii* PL. In the view of the above, in the present study was aimed to incorporate *B. licheniformis* at different serially diluted concentrations ($10^{-2}$ – $10^{-10}$) in formulated diets and fed to *M. rosenbergii* PL for assessing its optimum colony formation unit (CFU) for better activity of digestive enzymes, survival capacity, growth promoting ability and nutritional quantity of total protein, amino acid, carbohydrate and lipid. Its colonizing ability in the gut of *M. rosenbergii* and associated competitive exclusion of pathogenic bacteria was also studied.

## 2. MATERIALS AND METHODS

### 2.1. Procurement of *Bacillus licheniformis* (MTCC 429) and its sub-culture

The probiotic bacterium, *B. licheniformis* was procured from Microbial Type Culture Collection (MTCC 429 check the number), Chandigarh, India, in lyophilized powder form. The culture medium was prepared according to manufacturer's protocol (Table 1). The Nutrient broth (1.3 g) was mixed with double distilled water (100 ml) in a 100 ml sterile conical flask and autoclaved at

121 °C for 15 minutes. The probiotic *B. licheniformis* was inoculated into the broth and it was incubated for 24 hours at 37°C in a shaking incubator for its growth activity. The clear broth turned into turbid, which indicates the growth of *B. licheniformis* (Fig. 1). After incubation, the *B. licheniformis* cells were harvested by centrifugation (5000 rpm, 10 min), 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. 30µl of suspension was spread over the agar plate. The appearance of white colonies was observed (Fig.2). The broth was serially diluted up to $10^{-10}$ and 20 µl was spread on nutrient agar to enumerate the CFU in each dilution in order to optimize it, which showed presence 2580 colonies at $10^{-2}$ dilution, 1450 at $10^{-4}$, 935 at $10^{-6}$, 613 at $10^{-8}$, and 330 at $10^{-10}$ (Table 2).

**Table 1:** Composition of nutrient broth for mass culture and nutrient agar for sub-culture of *B. licheniformis*

| **Composition** | **g / Litre** |
|---|---|
| Peptic digest of animal tissue | 5.00 |
| Sodium chloride | 5.00 |
| Beef extract | 1.50 |
| Yeast extract | 1.50 |
| Agar* | 12.00 |
| Final pH (at 25 °C) | 7.4±0.2 |

**Fig 1:** Broth culture morphology of *A. licheniformis*

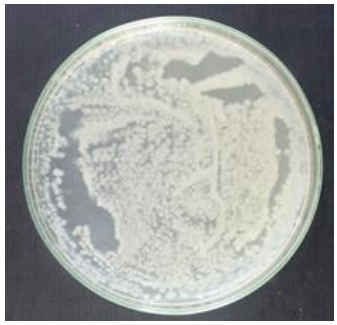

**Fig 2:** Spread plate culture morphology of *B. licheniformis* on nutrient agar

**Table 2:** CFU of *B. licheniformis* in nutrient agar plate culture at different serial dilution

| Serial Dilution | CFU |
|---|---|
| $10^{-2}$ | $2580x10^{-2}$ |
| $10^{-4}$ | $1450x10^{-4}$ |
| $10^{-6}$ | $935x10^{-6}$ |
| $10^{-8}$ | $613x10^{-8}$ |
| $10^{-10}$ | $330x10^{-10}$ |

**CFU**- Colony formation unit

### 2.2. Feed preparation

The branded feed basal ingredients, such as fish meal, groundnut oilcake, soybean meal, wheat bran, tapioca flour, sunflower oil and hen egg were purchased from local merchants at Coimbatore, India. Vitamin B-complex with vitamin-C (Pfizer Ltd., Mumbai, India) was purchased from local medical shop. The micro pulverized and sieved (0.3mm) basal ingredients, such as fishmeal (25%), groundnut oil cake (25%), and soybean meal (25%) were used as protein sources; 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 and thoroughly mixed **(Table 3).**The mixed feed ingredients were steam cooked for 15 min at 95-100°C and allowed to cool at room temperature. In such a way diets were prepared. Vitamin B-complex with vitamin C (1%) was added in the form of 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. Tapioca flour (5%) and egg albumin (7%) were used as binding agents, and sunflower oil (2%) was added as lipid source. Each serially diluted concentration of *B. licheniformis* ($10^{-2}$, $10^{-4}$, $10^{-6}$, $10^{-8}$ and $10^{-10}$) was separately incorporated. Thus, five such experimental feeds were formulated. The feed prepared without incorporation of *B. licheniformis* was served as control. Dough was prepared with 10% boiled water and pelletized in a manual pelletizer fixed with 3mm diameter and pellets were collected in aluminum trays. Then the feed was dried until the moisture content reached less than 10% under room temperature. The feed was physically examined for visual appearance, such as uniformity, color and fragrance. The pellets were with smooth surface. The prepared feed was subjected to analyses of proximate compositions and mineral contents. Feeds were prepared once in 15 days in order to maintain its viability.

**Table 3:** Proximate compositions and mineral contents in the basal diet formulated

| Proximate composition | (%) |
|---|---|
| Crude protein | 42.58 |
| Crude fat | 7.10 |
| Crude fibre | 0.72 |
| Ash | 7.53 |
| Moisture | 7.85 |
| Total nitrogen free extract | 32.54 |
| Sand and silica | 1.17 |
| Calcium | 0.85 |
| Phosphate | 0.93 |
| Iron | 0.14 |
| Copper | 0.001 |
| Manganese | 0.007 |
| Salt | 0.411 |

### 2.3. Viability of *B. licheniformis* in the formulated feeds

The diet was freshly prepared once in every 15 days to ensure the maintenance of high probiotic viability throughout the feeding trail. The viability of *B. licheniformis* in the feed was analyzed. One gram of each diluted concentration of *B. licheniformis* incorporated feed was taken and dissolved separately in autoclaved double distilled water (10 mL), they were serially diluted up to 10-6 then 20 µL of dilution was spread over nutrient agar medium, incubated at 37 ºC for 24 h and the colony morphology was observed, which was compared with the original *B. licheniformis* sub-culture morphology. Results revealed that *B. licheniformis* growth was identified in all culture plates except the control. Therefore, the incorporated *B. licheniformis* was viable even on day15 after the feed was formulated**.**

### 2.4. Procurement and acclimatization of experimental animal

The post larvae (PL-10) of the freshwater prawn, *M. rosenbergii* were procured from a prawn culture nursery pond, Marakkanam, Chennai, India. They were transported to the laboratory in polythene bags filled with oxygenated water. The prawns were acclimatized to the ambient laboratory condition in cement tanks with ground water for two weeks. The physicochemical parameters of diluents control water are presented in (Table 4). During acclimatization the prawns were fed with boiled egg albumin and artificially formulate feed (our laboratory prepared feed). More than 50% of tank water was routinely renewed every day in order to maintain a healthy environment and aeration was provided. This ensures sufficient oxygen supply to the prawns and an environment devoid of accumulated metabolic wastes. The unfed feeds, faecal material, exuvia

and moults, and dead prawns if any were removed by siphoning without disturbing the prawns.

**Table 4:** The physico-chemical parameters of the ground water

| Parameter | Value | Method |
|---|---|---|
| Temperature (°C) | 24 ± 0.3 °C | Mercury thermometer |
| pH | 7.4 ± 0.22 | pH meter |
| TDS (g/l) | 0.95 ± 0.06 | APHA, 2005 **[21]** |
| $DO_2$ (mg/l) | 4.13 ± 0.28 | Winkler method (1888) **[22]** |
| Salinity (mg/l) | 0.61 ± 0.02 | Water Analysis kit |
| EC (mS/cm) | 1.03± 0.02 | Water Analysis kit |
| Ammonia (mg/l) | 0.029 ± 0.005 | Solorzano (1969) **[23]** |

TDS, total dissolved solids; $DO_2$, dissolved oxygen; EC, Electrical conductivity.

### 2.4. Feeding trail

*M. rosenbergii* (≠ PL-25) ranging from 1.40±0.01cm in length and 0.10±0.04g in weight were used in this experiment. The prawns were divided into six experimental groups along with control, each group contained 40 PLs in 20L ground water and the experiment was conduct up to 90 days. In a triplicate experimental setup, five experimental groups were fed with $10^{-2}$, $10^{-4}$, $10^{-6}$, $10^{-8}$ and $10^{-10}$ serially diluted concentration of *B. licheniformis* incorporated diets, respectively. The control group was fed with basal diet formulated without incorporation of *B. licheniformis*. The unfed feed, exuvia and moults were siphoned out daily without severe disturbance to the prawn while renewing the water medium. On the $60^{th}$ day of experiment, assays of digestive enzymes activities were performed. On the $90^{th}$ day of experiment the survival rate was calculated and the morphometric data, such as the final length and weight were measured for evaluating the nutritional indices. The estimations of concentrations of basic biochemical constituents were also done.

#### 2.4.1. Assays of digestive enzymes

Activities of digestive enzymes were assayed at $60^{th}$ day of feeding trial. The digestive tract of three prawns from each replicate were carefully dissected and homogenized in ice-cold distilled water and centrifuged at 9000 g under 4 ºC for 20 min. The supernatant was used as a source of crude enzyme. Total protease activity was determined by casein-hydrolysis method of Furne *et al*., [24], where one unit of enzyme activity represented the amount of enzyme required to liberate 1 µg of tyrosine per minute. Amylase activity was determined according to Bernfeld *et al*., [25], the specific activity of amylase was calculated as milligrams of maltose liberated per gram of starch per hour (mg/g/h). Lipase activity was assayed by the method of Furne *et al*., [24], one unit of lipase activity was defined as the amount of free fatty acid released from triacylglycerol per unit time.

**2.4.2. Nutritional indices**

Nutritional indices, such as survival rate (SR), weight gain (WG), specific growth rate (SGR), feed conversion ratio (FCR) and protein efficiency rate (PER) were determined by following equations prescribed by Tekinay & Davis [26].

Survival (%) = Total No. of live animals/Total No. of initial animals × 100.

Weight gain (g) = Final weight (g) – Initial weight (g).

Specific growth rate (%) = log W2 – log W1/t ×100.

Where, W1 & W2 = Initial and Final weight, respectively (g), and t= Total number of experimental days.

Food conversion ratio (g) = Total Feed intake (g)/ Total weight gain of the prawn (g).

Protein efficiency ratio (g) = Total Weight gain (g)/ Total Protein consumed (g).

**2.4.3. Estimations of basic biochemical constituents**

The initial and final concentrations of total protein, amino acid and carbohydrate in experimental PL were estimated by adopting the methodology of Lowry *et al*., [27], Moore & Stein [28] and Roe [29] respectively. The total lipid was extracted by Folch *et al*., (30) method, and estimated gravimetrically by Barnes & Black Stock [31] method. The contents of ash and moisture were analysed by following AOAC [32] methodology.

**2.5. Gut microbial colonization**

The gut of control prawns and the gut of experimental prawns fed with the best concentration of *B. licheniformis* ($10^{-6}$) 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 flora. 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 $10^{-6}$ dilution individually. From this 0.5 mL of aliquots were taken and mixed with agar nutrient broth for 24 h at 35 ºC. 0.1 ml of broth culture was seeded over the surface of freshly prepared nutrient agar plates and incubated at 37 ºC for 24 h. The different bacterial colonies were identified and they were confirmed through routine bacteriological tests [33]. The following tests, such as 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 and carbohydrate fermentation test were performed. The bacterial colony was enumerated with the formula, Bacteria count (CFU/ g) = Number of colonies × Dilution factor/ Volume of sample (g).

**2.3.4. Statistical 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 respective tables. The *P*

values less than 0.05 were considered statistically (95%) significant.

## 3. RESULTS AND DISCUSSION

### 3.1. Digestive enzymes

Activities of digestive enzymes, such as protease, amylase and lipase were found to be significantly ($P< 0.05$) elevated in *B. licheniformis* supplemented diets fed prawns when compared with control diet fed prawns (Table 5). Among different concentrations, $10^{-6}$ of *B. licheniformis* supplementation produced the best performance. Digestive enzymes are among the most important factors that influence the efficiency of feed utilization by facilitating hydrolysis of carbohydrates, proteins and lipids present in feed [34]. The probiotics can influence digestive processes by enhancing the population of beneficial microorganisms, improving the intestinal microbial balance and microbial enzyme activity, consequently improving the digestibility and absorption of food and feed utilization [35], which was confirmed in this study by better growth performance and survival rate in *M. rosenbergii* PL fed with *B. licheniformis* supplemented diets. Similar enhancement of digestive enzymes activity has been reported in *M. rosenbergii* fed with probiotics incorporated feeds [8-11, 15-20, 36 - 38].

**Table 5:** Activities of digestive enzymes (U/ mg protein) in *M. rosenbergii* PL fed with *B. licheniformis* incorporated feeds.

| Parameter | Control | *B. licheniformis* | | | | |
|---|---|---|---|---|---|---|
| | | $10^{-2}$ | $10^{-4}$ | $10^{-6}$ | $10^{-8}$ | $10^{-10}$ |
| Protease | $2.01\pm0.09^{d}$ | $2.32\pm0.02^{c}$ | $2.98\pm0.15^{b}$ | $4.18\pm0.15^{a}$ | $2.51\pm0.21^{bc}$ | $2.12\pm0.25^{cd}$ |
| Amylase | $0.84\pm0.06^{de}$ | $2.32\pm0.04^{c}$ | $2.65\pm0.05^{b}$ | $3.02\pm0.03^{a}$ | $2.41\pm0.04^{bc}$ | $1.41\pm0.10^{d}$ |
| Lipase* | $0.24\pm0.02^{e}$ | $0.30\pm0.04^{c}$ | $0.39\pm0.03^{b}$ | $0.42\pm0.007^{a}$ | $0.35\pm.005^{bc}$ | $0.28\pm0.03^{cd}$ |

* U/ mg protein x $10^2$

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).

### 3.2. Nutritional indices

The survival rate (SR), growth performance (LG, WG and SGR) and PER were found to be significantly ($P< 0.05$) higher in *B. licheniformis* supplemented diets fed prawns when compared with control diet fed prawns (Table 6). Among different concentrations, $10^{-6}$ of *B. licheniformis* supplementation produced the best performance. The feed conversion ratio (FCR) was significantly ($P< 0.05$) lower in *B. licheniformis* supplemented diets fed prawns when compared with control diet fed prawns (Table 6). Among different concentrations, $10^{-6}$ of *B. licheniformis* supplementation produced the lowest FCR when compared with other supplemented concentrations of *B. licheniformis* and control diets fed prawns (Table 6). Similar nutritional indices have been reported in *M. rosenbergii* PL. [8-20, 36-38]. Nasim *et al.,* [39] reported that

dietary commercial probiotic *Bacilli* (*B. subtilis* and *B. licheniformis*) significantly improved the growth performance and feed utilization in *Litopenaeus vannamei.* Kolanchinathan *et al*., [40] reported that survival, growth performance were significantly increased in *Penaeus monodon* fed with diets containing of probiotic bacteria namely *B. coagulans* and *Bacillus firmus.* Zheng *et al.*, [42] reported that supplementation of *Lactobacillus plantarum* in diet had significantly improved growth performance of white shrimp, *L. vannamei*. Because, diet contained cell-free extract of *L. plantarum* produced the highest final body weight, weight gain rate and specific growth rate, and most efficient in terms of feed conversion.

**Table 6:** Nutritional indices of *M. rosenbergii* fed with *B. licheniformis* incorporated feeds (Initial morphometric data: Length, 1.40±0.01cm; Weight, 0.10±0.04g)

| **Parameters** | **Control** | ***B. licheniformis*** | | | | |
|---|---|---|---|---|---|---|
| | | **$10^{-2}$** | **$10^{-4}$** | **$10^{-6}$** | **$10^{-8}$** | **$10^{-10}$** |
| SR (%) | $76.66\pm3.33^{d}$ | $84.00\pm3.84^{bc}$ | $91.11\pm1.92^{a}$ | $94.44\pm1.92^{a}$ | $88.88\pm3.84^{ab}$ | $78.88\pm5.09^{cd}$ |
| Length(cm) | $1.73\pm0.05^{d}$ | $2.40\pm0.10^{c}$ | $2.76\pm0.15^{b}$ | $3.36\pm0.15^{a}$ | $2.6\pm0.10^{bc}$ | $2.10\pm0.10^{c}$ |
| Weight (g) | $0.32\pm0.02^{f}$ | $0.65\pm0.03^{d}$ | $1.16\pm0.02^{b}$ | $1.32\pm0.08^{a}$ | $0.93\pm0.01^{c}$ | $0.53\pm0.03^{e}$ |
| LG (cm) | $0.33\pm0.05^{e}$ | $1.00\pm0.20^{cd}$ | $1.36\pm0.20^{b}$ | $1.96\pm0.20^{a}$ | $1.20\pm0.17^{bc}$ | $0.70\pm0.17^{d}$ |
| WG (g) | $0.25\pm0.02^{f}$ | $0.57\pm0.03^{d}$ | $1.08\pm0.01^{b}$ | $1.24\pm0.08^{a}$ | $0.85\pm0.01^{c}$ | $0.45\pm0.03^{e}$ |
| SGR (%) | $0.75\pm0.04^{e}$ | $1.05\pm0.04^{c}$ | $1.31\pm0.02^{a}$ | $1.36\pm0.04^{a}$ | $1.21\pm0.02^{b}$ | $0.97\pm0.04^{d}$ |
| FCR (g) | $27.29\pm2.42^{a}$ | $12.33\pm0.70^{c}$ | $7.09\pm0.12^{d}$ | $6.57\pm0.47^{d}$ | $8.27\pm0.16^{d}$ | $16.91\pm1.20^{b}$ |
| PER (g) | $0.08\pm0.007^{f}$ | $0.18\pm0.01^{d}$ | $0.32\pm0.005^{b}$ | $0.34\pm0.02^{a}$ | $0.28\pm0.01^{c}$ | $0.13\pm0.009^{e}$ |

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.

### 3.3. Biochemical constituents

Concentrations of basic biochemical constituents, such as total protein, amino acid, carbohydrate, lipid and ash were found to be significantly ($P< 0.05$) elevated in *B. licheniformis* supplemented diets fed prawns when compared with control. Among different concentrations, $10^{-6}$ of *B. licheniformis* supplemented diets produced significantly the best performance (Table 7). Similar nutritional indices have been reported in *M. rosenbergii* PL [8-20, 36-38]. It has been reported that the biochemical constituents and immune expression were increased significantly in the shrimp, *P. monodon* fed with diets containing *B. coagulans* and *B. firmus* individually and combined upon infection with *Vibrio alginolyticus* [42] Further, the mean weight gain, mean length gain, SGR, mean feed intake and survival rate were increased, and reverse in the FCR was recorded.

**Table 7:** Concentrations of basic biochemical constituents (mg/g wet wt.) in *M. rosenbergii* PL fed with *B. licheniformis* incorporated feeds

| Parameter | Control | *B. licheniformis* | | | | |
|---|---|---|---|---|---|---|
| | | $10^{-2}$ | $10^{-4}$ | $10^{-6}$ | $10^{-8}$ | $10^{-10}$ |
| Protein | 62.12±2.78$^{f}$ | 129.23±1.41$^{d}$ | 152.00±2.17$^{b}$ | 171.12±1.48$^{a}$ | 140.23±1.20$^{c}$ | 100.25±0.90$^{e}$ |
| Amino acid | 32.44±1.25$^{f}$ | 50.25±1.47$^{d}$ | 65.25±0.95$^{b}$ | 71.40±1.23$^{a}$ | 62.52±1.28$^{bc}$ | 41.25±2.12$^{e}$ |
| Carbohydrate | 18.32±0.85$^{f}$ | 28.00±1.58$^{d}$ | 37.75±1.42$^{b}$ | 42.25±1.41$^{a}$ | 33.25±0.96$^{c}$ | 23.52±0.56$^{e}$ |
| Lipid | 15.38±0.45$^{f}$ | 28.70±0.14$^{d}$ | 37.45±0.63$^{b}$ | 40.12±0.45$^{a}$ | 33.45±0.58$^{c}$ | 20.41±0.56$^{e}$ |
| Moisture (%) | 85.65±2.41$^{a}$ | 77.20±2.52$^{c}$ | 73.14±3.27$^{d}$ | 70.23±3.37$^{e}$ | 75.12±2.58$^{cd}$ | 80.56±1.29$^{b}$ |
| Ash (%) | 14.97±1.45$^{f}$ | 22.12±2.58$^{c}$ | 26.12±1.85$^{a}$ | 18.45±1.89$^{de}$ | 24.53±2.23$^{b}$ | 19.56±2.52$^{d}$ |

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).

### 3.4. Gut microbes

In the gut of control prawns, presence of *Pesudomonas* sp., *Klebseilla* sp., *Escharichia coli, Bacillus* sp., *Streptococcus* sp., *Staphylococcus* sp., *Citrobacter* sp., and *Acinitobacter* sp., (96.92%) were identified through colony morphology and biochemical tests (Table 8). In the gut of PL fed with $10^{-6}$ of *B. licheniformis* showed presence of *Bacillus* sp., *Bacillus* sp., *Lactobacillus* sp., *E. coli, Streptococcus* sp., and *Citrobacter* sp., were identified through colony morphology and biochemical tests. It was found to be competitively excluded few pathogenic bacteria such as *Pesudomonas* sp., *Klebseilla pneumonia, Staphylococcus* sp., and *Acinitobacter* sp., from the gut of experimental prawns fed with $10^{-6}$ of *B. licheniformis*. It was also found that additional establishment of one *Lactobacillus* sp., and one *Bacillus* sp., were identified in the gut of experimental prawns, which revealed the establishment of probably *B. licheniformis* (Table 8). The confirmative results of biochemical tests for micro flora present in the gut of *M. rosenbergii* fed with control and $10^{-6}$of *B. Licheniformis* supplemented diets are presented in tables 9 and 10, respectively. Competitive exclusion of pathogenic bacteria, *Pseudomonas aeruginosa*, *K. pneumonia*, *Acetonobacter* sp., and *Salmonella* sp., by probiotics, like *L. fermentum*, *L. brevis*, *B. coagulans* and *B. subtilis* supplemented feed fed *M. rosenbergii* have reported [8-20, 36-38] that an increase in *Lactobacillus* sp., and *Bacillus* sp., counts and decrease in *Vibrio* sp., in *Lactobacillus lactis* supplemented feed fed *L. vannamei*, it was an indication of the positive role of this potential probiotic in improving the growth and feed efficiency. Venkat *et al.*, [43] reported that *M. rosenbergii* fed with *Lactobacillus* sp., supplemented feed showed significant decrease of pathogenic bacteria (*Enterobacteriacea*., *Aeromonas* sp., and *Pseudomonas* sp.). It is valid to mention here that 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 [44-48]. The presence of at least 10 bacterial genera *E. coli*, *Proteus* sp., *Lactococcus* sp., *Enterobacteria* sp., *Lactobacillus* sp., *Pseudomonas* sp., *Staphylococcus* sp., *Bacillus* sp., *Klebsiella* sp., *Streptococcus* sp., have been isolated from the gut of *Oreochromis niloticus* and *Clarias gariepinus* [49].

**Table 8:** Microbial load in the gut of *M. rosenbergii* PL fed with control and $10^{-6}$of *B. licheniformis* supplemented diets

| Samples | Isolated species | Composition (%) |
|---|---|---|
| Control | *Pesudomonas* sp., | 10.21 |
| | *Klebseilla* sp., | 11.78 |
| | *Escharichia coli* | 12.45 |
| | *Bacillus* sp., | 12.25 |
| | *Streptococcus* sp., | 9.89 |
| | *Staphylococcus* sp., | 11.89 |
| | *Citrobacter* sp., | 12.45 |
| | *Acinitobacter* sp., | 13.33 |
| | Total | 96.92% |
| *B. licheniformis* | *Bacillus* sp, | 18.81 |
| | *Bacillus* sp., | 12.50 |
| | *Lactobacillus* sp., | 12.02 |
| | *Escharichia coli* | 13.89 |
| | *Streptococcus* sp., | 15.56 |
| | *Citrobacter* sp., | 17.58 |
| | Total | 90.36 |

**Table 9:** Confirmative results of biochemical tests for microflora present in the gut of *M. rosenbergii* PL fed with control diet

| Test | A | B | C | D | E | F | G | H |
|---|---|---|---|---|---|---|---|---|
| Gram's stain | - | - | - | + | + | + | - | - |
| Motility Test | M | NM | M | M | NM | M | M | M |
| Capsule | NC | C | V | C | C | NC | C | NC |
| Spore | NS | NS | NS | S | NS | NS | S | NS |
| Flegella | SF | NF | F | F | NF | F | F | F |
| Indole Test | - | - | + | - | - | - | - | - |
| Methylred Test | - | - | + | - | + | + | + | + |
| Vp Test | - | + | - | + | - | + | - | - |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Citrate Utilization Test | + | + | - | + | + | + | + | + |
| Urase Test | - | + | - | - | - | + | - | V |
| Starch Hydrolases Test | + | - | - | + | - | - | - | - |
| Gelatin Hydrolases | + | - | + | + | + | + | - | - |
| Nitrate Reduction Test | - | + | + | + | - | + | + | + |
| Oxidase Test | + | - | - | - | - | + | - | - |
| Reduction Test | - | + | + | - | - | - | - | - |
| Catalase Test | + | + | - | + | - | + | + | + |
| Glucose Test | A | A | A | A | A | A | A | NA |
| Lactose Test | NA | A | A | NA | A | A | A | A |
| Sucrose Test | A | A | NA | A | A | A | A | A |
| Mannitol Test | A | A | A | A | NA | A | NA | A |
| Maltose Test | A | A | NA | A | A | A | A | A |

M, Motile; NM, Non-Motile; NC, No Capsule; C, Capsulated; V, Variable; NS, Non Sporulated; S, Sporulated; SF, Single flagella; F, Flagella; NF, No flagella; A, Acid production; NA, No Acid production; "-", Negative; "+", Positive. **A**, *Pesudomonas* sp.; **B**, *Klebseilla* sp.; **C**, *E. coli*; **D**, *Bacillus* sp.; **E**, *Streptococcus* sp.; **F**, *Staphylococcus* sp.; **G**, *Citrobacter* sp.; **H**, *Acinitobacter* sp.

**Table 10:** Confirmative results of biochemical tests for microflora present in the gut of *M. rosenbergii* fed with $10^{-6}$of *B. licheniformis* supplemented diet

| **Test** | **A** | **B** | **C** | **D** | **E** | **F** |
|---|---|---|---|---|---|---|
| Gram's stain | - | - | + | + | + | - |
| Motility Test | M | M | M | NM | M | M |
| Capsule | V | V | C | C | C | C |
| Spore | NS | NS | S | NS | NS | S |
| Flegella | F | F | F | NF | NF | F |
| Indole Test | + | + | - | - | - | - |
| Methylred Test | + | + | - | + | - | + |
| Vp Test | - | - | + | - | - | - |
| Citrate Utilization Test | + | - | + | + | - | + |
| Urase Test | - | - | - | - | - | - |
| Starch Hydrolases Test | - | + | + | - | + | - |
| Gelatin Hydrolases | + | + | + | + | + | - |
| Nitrate Reduction Test | + | + | + | - | + | + |
| Oxidase Test | - | - | - | - | - | - |

| | | | | | | |
|---|---|---|---|---|---|---|
| Reduction Test | + | + | - | - | - | - |
| Catalase Test | - | - | + | - | - | + |
| Glucose Test | A | A | A | A | A | A |
| Lactose Test | A | A | NA | A | A | A |
| Sucrose Test | NA | NA | A | A | A | A |
| Mannitol Test | A | A | A | NA | A | NA |
| Maltose Test | NA | NA | A | A | A | A |

M, Motile; NM, Non-Motile; NC, No Capsule; C, Capsulated; V, Variable; NS, Non Sporulated; S, Sporulated; SF, Single flagella; F, Flagella; NF, No flagella; A, Acid production; NA, No Acid production; "-", Negative; "+", Positive. **A**, *Bacillus* sp.; **B**, *Bacillus* sp.; **C**, *Lactobacillus* sp.; **D**, *E. coli*.; **E**, *Streptococcus* sp.; **F**, *Citrobacter* sp.

## 4. CONCLUSION

For bacteria to be considered as probiotic it must be capable of surviving passage through the gastrointestinal tract. It must then colonize the host digestive system, either by adhering to the mucous membrane surface or the intestinal epithelia. Finally, it must be able to produce inhibitory or antagonist metabolites against undesirable native flora, and reproduce **[50-52].** As *B. licheniformis* possessed these qualities, it could serve as a potential feed additive. In this study, *B. licheniformis* supplemented feed, particularly at $10^{-6}$ (CFU= $935x10^{-6}$) has colonized in the gut of *M. rosenbergii* PL, and competitively excluded *Pesudomonas* sp, *K. pneumonia* sp., *Staphylococcus* sp, and *Acinitobacter* sp. Thus it is suggested that this probiotic possessed immunomodulatory property and improving the general health of the prawn. The increased activity of protease, amylase and lipase have improved the digestion of protein, starch and fat, respectively, which in turn led to proper absorption and assimilation of food that serves as the reason for better FCR, survival, growth and production of quantum of protein, amino acid, carbohydrate and lipid observed. Therefore, *B. licheniformis* is recommended for sustainable culture of *Macrobrachium*.

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## CONFLICT OF INTEREST

The authors have no conflict of interest.

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