

Available online at [GSC Online Press Directory](#)

GSC Biological and Pharmaceutical Sciences

e-ISSN: 2581-3250, CODEN (USA): GBPSC2

Journal homepage: <https://www.gsconlinepress.com/journals/gscbps>

(RESEARCH ARTICLE)



## Growth and survival promotion of a probiotic bacterium *Enterococcus durans* enriched *Artemia* nauplii on the prawn *Macrobrachium rosenbergii*

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Publication history: Received on 23 June 2020; revised on 06 July 2020; accepted on 08 July 2020

Article DOI: <https://doi.org/10.30574/gscbps.2020.12.1.0201>

### Abstract

This work accentuate the growth performance of *Macrobrachium rosenbergii* post larvae (PL) fed with a probiotic bacterium, *Enterococcus durans* enriched live feed *Artemia franciscana* nauplii at five different serially diluted concentrations ( $10^{-1}$ ,  $10^{-3}$ ,  $10^{-5}$ ,  $10^{-7}$  and  $10^{-9}$ ). After 45 days of the feeding trial, the survival rate, growth rate, activities of protease, amylase and lipase, and contents of total protein, amino acid, carbohydrate and lipid were significantly ( $P < 0.05$ ) increased, particularly at  $1071 \times 10^{-7}$  CFU. The gut microflora of un-enriched *Artemia* nauplii fed prawns showed the presence of *Escherichia coli*, *Klebsiella* sp., *Citrobacter* sp., *Acinetobacter* sp., *Streptococcus* sp., *Bacillus* sp., *Staphylococcus* sp., and *Pseudomonas* sp. The optimized concentration of *E. durans* enriched *Artemia* nauplii fed prawns showed the presence of *Enterococcus* sp., *E. coli*, *Bacillus* sp., and *Klebsiella* sp. The pathogenic bacteria, *Citrobacter* sp., *Acinetobacter* sp., *Streptococcus* sp., *Staphylococcus* sp., and *Pseudomonas* sp., were competitively excluded in the gut of prawns due to the colony establishment of *Enterococcus* sp., Hence, *E. durans* can be used as a enrichment material of *Artemia* nauplii for sustainable culture of *M. rosenbergii*.

**Keywords:** Live feed; *Artemia*; Prawn; Probiotics; *E. durans*; Growth; Survival; Gut microflora; Digestive enzymes; Protein

### 1. Introduction

Aquaculture is one of the fastest growing food producing sectors in the world. It offers proteinaceous food supply to fight against malnutrition, poverty and considerably generate employment opportunity to improve rural economy. In recent years both seafood production and overall worldwide consumption has increased considerably [1]. Among crustacean, prawns provide protein, vitamins, minerals and nutritious delicacy for human consumption [2]. The growth of aquaculture has resulted in environmental damages, which can be minimized through sustainable practices. The use of antibiotics and chemicals can be reduced and the outbreaks of diseases can be prevented by applications of probiotics [3, 4].

In aquaculture, probiotics has been extended in feed supplements, have the ability to survive and pass through the intestinal tract. They have competitively exclude pathogenic bacteria by production of inhibitory compounds in an eco-friendly manner, increase immune response, stress resistance, disease tolerance, maintain water quality, encourage reproduction and offer better growth and survival [5-20]. Probiotics increase digestibility of nutrients and feed efficiency by producing some exogenous enzymes, which increases activities of endogenous enzymes (amylase, protease and lipase) to hydrolyze nutrients, and effectively improve growth, survival and nutritional quality of the host [21-30].

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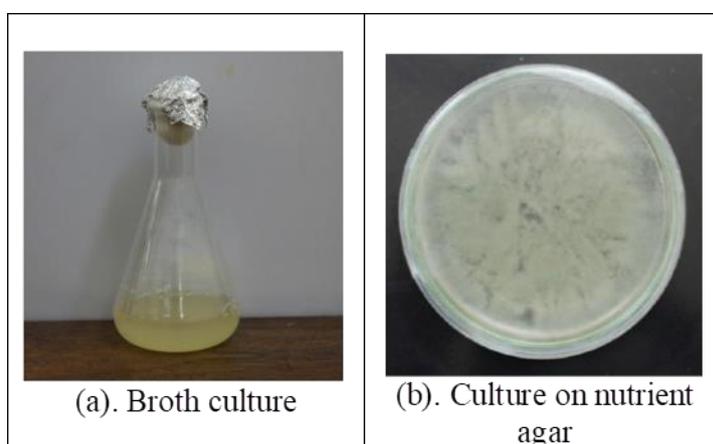
The genus *Enterococcus* is the most controversial group of lactic acid bacteria. *Enterococci* are ubiquitous microorganisms, widely distributed in nature (water, plant, soil, foods, and gastrointestinal tract of humans and animals) and considered as bacteria of low pathogenicity. *Enterococcus durans*, *Enterococcus faecium*, *Enterococcus faecalis* and *Enterococcus avium* produces antimicrobial bacteriocins called enterocins, they used in food biopreservation, play an important role in the ripening of cheeses, probably through proteolysis, lipolysis, and citrate breakdown. *Enterococci* are nowadays have shown a very appreciable probiotic characteristics in animal nutrition and used as potential probiotic candidates in aquaculture, live cells with different beneficial characteristics such as stimulation of immunity, anti-inflammatory activity, hypocholesterolemic effect, and prevention/treatment of some diseases in hosts. At the same time, *Enterococci* have been associated with a number of human infections [31-35].

It has been reported that high ammonia and high dissolved oxygen in water decreased the virulence of *Enterococcus* in *Macrobrachium rosenbergii* culture medium [36, 37], and thus, *Enterococcus* can be used as probiotics. *Enterococcus* genus is isolated from the intestine of common carp and freshwater prawn *M. rosenbergii* [38]. *Enterococcus gallinarum* and *Enterococcus hirae* have proved with probiotic functions in *M. rosenbergii* [39, 40] when ingested in sufficient quantities. *E. durans* possessed quorum quenching activity (ability to grow in low pH) and to produce antimicrobial agents, hence, this bacterium could be used for therapeutics [31, 41]. Therefore in this study, *E. durans* was used as a probiotic bacterium to enrich live *Artemia* nauplii for feeding *M. rosenbergii* PL in order to assess the ability of *E. durans* to increase the survival and growth, activities of digestive enzymes (amylase, protease and lipase) and the contents of basic biochemical constituents (total protein, total amino acid, total carbohydrate and total lipid).

## 2. Material and methods

### 2.1. Procurement of *Enterococcus durans* (3031) and its sub culture

The lyophilized spores of *E. durans* (MTCC 3031) was purchased from Microbial Type Culture Collection (MTCC), Chandigarh, India. It was subjected to sub-culture with Nutrient broth (Hi-media, India, pH, 6.5 at Temperature, 25 °C), contained peptic digestion of animal tissues (5 g L<sup>-1</sup>), Beef extract (1.5 g L<sup>-1</sup>), Sodium chloride (5.0 g L<sup>-1</sup>), and Yeast extract (1.5 g L<sup>-1</sup>). The medium (13 g) was mixed with 1 L of double distilled water in a screw cap container and autoclaved at 121°C for 15 minutes. A loop full of *E. durans* was inoculated into the broth and incubated for 24 hours at 37°C. The appearance of turbid broth indicates the growth of *E. durans* (Figure 1). The cultured *E. durans* was harvested by centrifugation at 5000 rpm for 10 min, washed twice with phosphate buffered saline (pH, 7.2), weighed and re-suspended in the same buffer. For further usage it was stored at 4 °C. The suspension (30µl) was spread over the agar plate and the appearance of white colony indicates the growth of *E. durans* (Figure 1). 20 µl of serially diluted broth (up to 10<sup>-9</sup>) was spread on nutrient agar for enumerating the CFU in order to optimize it, and the count was 3874 at 10<sup>-1</sup>, 2671 at 10<sup>-3</sup>, 1864 at 10<sup>-5</sup>, 1071 at 10<sup>-7</sup>, and 684 at 10<sup>-9</sup>.



**Figure 1** Culture morphology of *E. durans*

### 2.2. Feed preparation

All the ingredients used were micro pulverized and sieved (0.3 mm). For protein source, fishmeal (25%), groundnut oil cake (25%) and soybean meal (25%) were used. For carbohydrate source, wheat bran (10%) was used. Then it was steam cooked for 15 min at 95-100°C and cool at room temperature. BECOSULES CAPSULES (Pfizer Ltd., Mumbai, India)

was used for Vitamin B complex with vitamin C (1%). Tapioca flour (5%) and egg albumin (7%) were used as binding agents. Sunflower oil (2%) was added as lipid source. The dough was prepared with adequate boiled water, pelletized in a manual pelletizer fixed with 3 mm diameter metal mesh, the threads were collected in aluminum trays and the semidried threads were cut into 3-5 mm pellets. The pelletized feed was dried under room temperature until the moisture content reached less than 10%. The prepared feed was subjected to proximate composition analysis by adopting AOAC (1995) methodology. The feed proximate composition was as follows: Crude protein, 46.79; Total Nitrogen-free extract, 32.21; Ether extract (Crude fat), 6.19; Crude fiber, 1.33; Ash, 6.81; Moisture, 9.84; Gross energy, 4443 kcal/kg [39, 40]. The feed mineral content (%) was as follows: Sand and silica (Acid insoluble ash), 0.83; Calcium, 0.80; Phosphorus, 0.90; Iron, 0.11; Copper, 0.002; Manganese, 0.008; Salt, 0.58 [39, 40].

### 2.3. Enrichment of *Artemia nauplii* with *E. durans*

The brine shrimp, *Artemia franciscana* cyst was purchased from Aqua world, Paris Corner, Chennai, India. The cysts (2 g/ 20 L and 15 g kg<sup>-1</sup> body biomass of the prawns) were taken and hydrated in 1 L<sup>-1</sup> of purified artificial saltwater (prepared from artificial sea salt powder 35.0 g L<sup>-1</sup>, pH of 6.5) for 12-15 h. The cysts burst and the embryo surround by the hatching membrane become visible for few hours. The brownish orange colored nauplii came out. The 48-hr old *Artemia* nauplii were filtered and transferred to 1 L capacity glass beaker. Five such groups were enriched with 3874×10<sup>-1</sup>, 2671×10<sup>-3</sup>, 1864 ×10<sup>-5</sup>, 1071×10<sup>-7</sup>, and 684×10<sup>-9</sup> concentrations of *E. durans* for 1 hour (Figure 2). The *Artemia* nauplii were washed with freshwater and fed to *M. rosenbergii* PL.

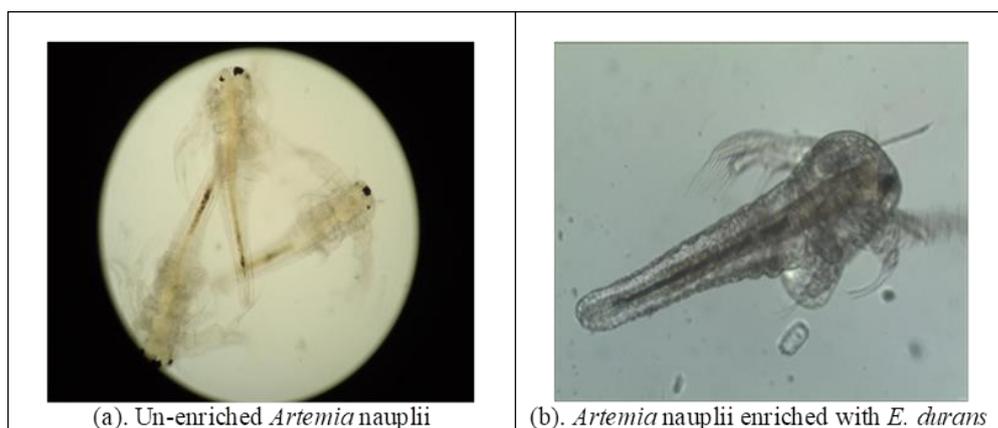


Figure 2 *A. franciscana* nauplii (24 hrs old)

### 2.4. Procurement and acclimatization of experimental animal

The post larvae (PL-8) of *M. rosenbergii* were procured from a prawn hatchery, Marakkanam, Chennai, India. They were transported to the laboratory in polythene bags filled with oxygenated water and acclimatized with ground water (Temperature, 28±2.2 °C; pH, 7.4±0.10; TDS, 0.94±0.05 g/L; DO 4.25±0.25 mg/L; Salinity, 0.70±0.02 mg/L; EC, 1.01±0.01 Ms/cm; Ammonia, 0.028±0.006 mg/L) for two weeks in cement tanks [1, 2]. During acclimatization the prawns were fed with *Artemia* nauplii, boiled egg albumin threads and artificially feed formulated in our laboratory. Nearly half of the tank water was renewed every day and adequately aerated in order to maintain a healthy environment. The unfed feed, faecal material, exuvia/moult, and dead prawns if any were routinely removed by siphoning without disturbing the prawns while renewing the water medium [39, 40].

### 2.5. Feeding trial

The feeding trials were conducted for a period of 45 days. Seven group of *M. rosenbergii* (1.1±0.05 cm and 0.05±0.003 g) were taken. The group 1 was fed with artificial feed, group 2 was fed with unenriched *Artemia* nauplii, and groups 3-7 were fed with *E. durans* (3874×10<sup>-1</sup>, 2671×10<sup>-3</sup>, 1864 ×10<sup>-5</sup>, 1071×10<sup>-7</sup>, and 684×10<sup>-9</sup>) enriched *Artemia* nauplii. Each group comprised of 30 individuals accommodated in 25L of ground water. The water medium was renewed daily by siphoning and aerated. While renewing the water medium, the unfed feed, feces and moult were removed. At the end of the feeding trial the morphometric measurements were taken for calculating the nutritional indices and estimating concentrations of basic biochemical constituents, such as total protein, amino acid, carbohydrate and lipid, and activities of digestive enzymes, such as protease, amylase and lipase.

## 2.6. Calculation of nutritional indices

The survival rate (SR), length gain (LG), weight gain (WG), and specific growth rate (SGR) were individually calculated [42].

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

Length gain (cm) = Final length (cm) – Initial length (cm)

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

Specific growth rate, (%) =  $\log W_2 - \log W_1 / t \times 100$

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

## 2.7. Estimations of basic biochemical constituents

The basic biochemical constituents, such as total protein, amino acid and carbohydrate were estimated in test prawns adopting standard methodologies [43-45], respectively, and the total lipid was extracted gravimetrically [46] and spectrophotometrically estimated [47]. The contents of ash and moisture were analysed [48].

## 2.8. Assays of digestive enzymes activities

Activities of digestive enzymes (protease, amylase and lipase) were assayed at 45<sup>th</sup> day of feeding trial. The digestive tract of three prawns from each replicate were carefully dissected out and homogenized in ice-cold distilled water and centrifuged at 9000 g under 4 °C 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., [49], 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 [50], 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., [49], one unit of lipase activity was defined as the amount of free fatty acid released from triacylglycerol per unit time.

## 2.9. Gut microbial colonization

The bacterial culture was performed in the gut homogenate of experimental prawns fed with *E. durans* ( $1071 \times 10^{-7}$ ). The prawns were deactivated by kept them in freezer at -20 °C for 10 minutes. The surface of the prawn was sterilized with 50 ppm formalin for 30 seconds for removing external flora. Then the digestive tract was dissected out and homogenized with phosphate buffered saline (pH, 7.2) under aseptic condition. The homogenate was serially diluted up to  $10^{-7}$ . The aliquot (0.5 mL) was mixed with agar nutrient broth and incubated at 35 °C for 24 h. The broth (0.1 ml) was seeded on the surface of freshly prepared nutrient agar plate and incubated at 37 °C for 24 h. Different bacterial colonies seen were identified and confirmed through routine bacteriological 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 [51]. The bacterial colony was enumerated by using the formula, Bacterial count (CFU/ g) = Number of colonies × Dilution factor/ Volume of sample (g).

## 2.10. Statistical analysis

All the data were subjected to statistical analysis through one-way ANOVA and subsequent post-hoc multiple comparisons (DMRT using SPSS v20). The *P* value less than 0.05 was considered as statistically (95%) significant.

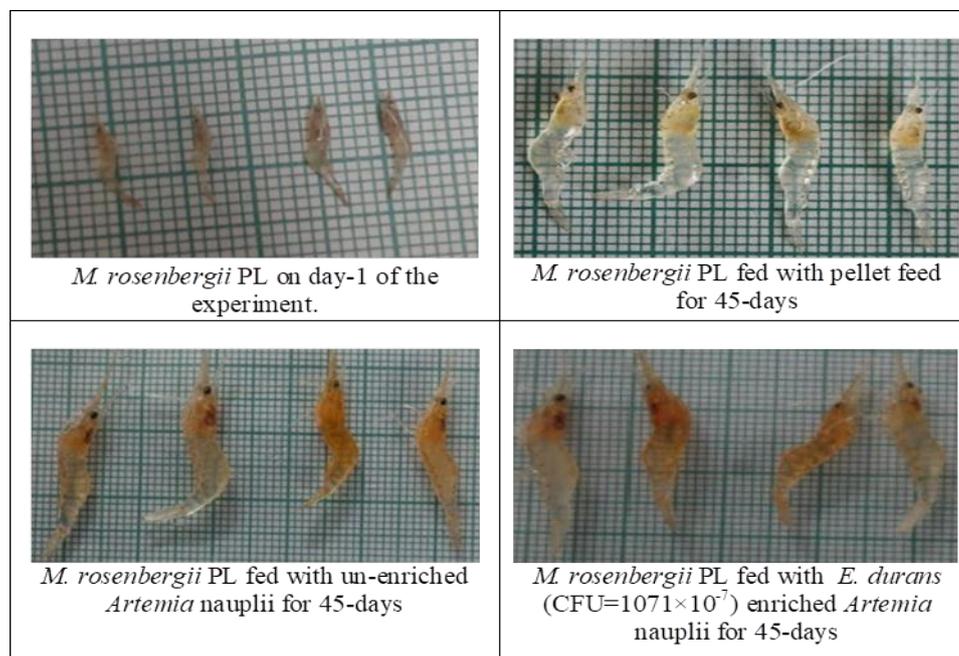
## 3. Results and discussion

### 3.1. Survival rate and nutritional indices

The SR, WG and SGR were found to be significantly ( $P < 0.05$ ) higher in all concentrations of *E. durans* ( $3874 \times 10^{-1}$ ,  $2671 \times 10^{-3}$ ,  $1864 \times 10^{-5}$ ,  $1071 \times 10^{-7}$ , and  $684 \times 10^{-9}$ ) enriched *Artemia* nauplii fed *M. rosenbergii* PL when compared with un-enriched *Artemia* nauplii and the PL fed with formulated artificial feed. Among these concentrations,  $1071 \times 10^{-7}$  was produced the best growth and survival performances (Table 1; Figure 3).

Similar enhanced growth performances have been reported by following workers. Jain et al., [39, 40] in *M. rosenbergii* PL fed with *E. gallinarum* and *E. hirae* enriched *Artemia* nauplii. Seenivasan et al., [52] in *M. rosenbergii* fed with *Lactobacillus sporogenes* enriched *Artemia* nauplii. Ali et al., [53] in *Litopenaeus vannamei* PL fed with *Chaetoceros gracilis* and *Saccharomyces cerevisiae* enriched *Artemia* nauplii. Mahmood et al., [54] in Angelfish, *Pterophyllum scalare* fed with symbiotic bacterium, *Pediococcus acidilactici* and fructo-oligosaccharide enriched *Artemia* nauplii. Nadella et al., [55, 56] reported that *Bacillus licheniformis* significantly reduced the cumulative mortality rate of *M. rosenbergii*

challenged with *Vibrio alginolyticus*, due to improved immunity. Liu et al., [18] reported a significant increase in weight gain in parrot fish, *Oplegnathus fasciatus* fed with the diet containing *B. subtilis* E20 and disease resistance against *V. alginolyticus*. Liu et al., [57] recorded that *Bacillus* sp., administration has increased the survival rate of kuruma shrimp, *Marsupenaeus japonicas*. The enhanced performance have also been reported in shrimp *L. vannamei* fed with microencapsulated and freeze-dried *Bacillus* [58], in *M. rosenbergii* fed with *Bacillus coagulans*, Binifit™, LactoBasil®<sub>plus</sub>, ViBact\*, *B. subtilis* and *B. licheniformis* supplemented feeds [21, 22, 26, 30, 59-61].



**Figure 3** *M. rosenbergii* PL at initial and end of the experiment

### 3.2. Activities of digestive enzymes

Activities of protease, amylase and lipase were found to be significantly ( $P < 0.05$ ) elevated in *E. durans* enriched *Artemia* nauplii fed prawns when compared with un-enriched *Artemia* nauplii and artificial pelletized feed. Among the different concentrations of *E. durans*,  $1071 \times 10^{-7}$  has produced the best performance (Table 2).

Similar elevations in activities of protease, amylase and lipase have been reported by Jain et al., [39, 40] in *M. rosenbergii* PL fed with *E. gallinarum* and *E. hirae* enriched *Artemia* nauplii. Elevated activities of protease, amylase and lipase have also been recorded in *M. rosenbergii* PL fed with LactoBasil®<sub>plus</sub>, ViBact\*, *L. sporogenes*, *Bacillus subtilis*, *S. cerevisiae*, *Lactobacillus brevis*, *Lactobacillus fermentum*, *B. coagulans* and *B. licheniformis* supplemented feeds [21, 22, 24-30]. It has been reported that a significant increase in activities of digestive enzymes in *Penaeus vannamei* larvae when using *B. coagulans* and *B. subtilis* in its feeding regime [62-64]. The increases in activities of digestive enzymes have also been reported in several fish spp., when administered various probiotics: in grouper, *Epinephelus coioides*, *Epinephelus lanceolatus* and *Epinephelus fuscoguttatus* when supplemented with *Lactococcus lactis*, *E. faecium*, *Bacillus cereus*, *Lactobacillus acidophilus* and *Clostridium butyricum* [65, 66]; in the catfish, *Clarias* sp., against *Bacillus megaterium* [67]; in *Tor grypus* against *Lactobacillus bulgaricus* and *Lactobacillus plantarum* [68]; in the common carp, *Cyprinus carpio* against *Lactobacillus delbrueckii* [69].

### 3.3. Contents of basic biochemical constituents

Concentrations of total protein, amino acids, carbohydrate, and lipids were found to be significantly ( $P < 0.05$ ) elevated in *E. durans* enriched *Artemia* nauplii fed prawns when compared with un-enriched *Artemia* nauplii and artificial pelletized feed. Among the different concentrations of *E. durans*,  $1071 \times 10^{-7}$  has produced the best performance (Table 3).

**Table 1** Survival and growth of *M. rosenbergii* PL fed with pelletized artificial feed, un-enriched *Artemia* nauplii and *E. durans* enriched *Artemia* nauplii for 45-days

Parameter	Pelletized Artificial feed fed PL. Jain et al., [39, 40]	Un-enriched <i>Artemia</i> nauplii fed PL. Jain et al., [39, 40]	Different concentrations of <i>E. durans</i> enriched <i>Artemia</i> nauplii fed PL					F-value
			3874×10 <sup>-1</sup> CFU	2671×10 <sup>-3</sup> CFU	1864×10 <sup>-5</sup> CFU	1071×10 <sup>-7</sup> CFU	684×10 <sup>-9</sup> CFU	
SR (%)	71.77±1.92 <sup>f</sup>	76.66±3.37 <sup>e</sup>	80.00±3.33 <sup>de</sup>	84.44±1.93 <sup>cd</sup>	90.00±3.33 <sup>ab</sup>	92.22±3.84 <sup>a</sup>	86.66±3.33 <sup>ab</sup>	17.85
Length(cm)	1.33±0.06 <sup>f</sup>	1.67±0.06 <sup>e</sup>	1.90±0.1 <sup>ef</sup>	2.11±0.12 <sup>cd</sup>	2.34±0.34 <sup>bc</sup>	3.08±0.11 <sup>a</sup>	2.57±0.11 <sup>b</sup>	40.98
Weight (g)	0.49±0.07 <sup>e</sup>	0.72±0.05 <sup>cd</sup>	0.90±0.08 <sup>cd</sup>	1.17±0.28 <sup>bc</sup>	1.30±0.37 <sup>abc</sup>	1.62±0.31 <sup>a</sup>	1.36±0.12 <sup>bc</sup>	31.44
LG (cm)	0.33±0.11 <sup>f</sup>	0.66±0.11 <sup>e</sup>	0.90±0.17 <sup>ef</sup>	1.11±0.10 <sup>cd</sup>	1.34±0.39 <sup>bc</sup>	2.08±0.05 <sup>a</sup>	1.57±0.05 <sup>b</sup>	9.58
WG (g)	0.45±0.06 <sup>e</sup>	0.67±0.05 <sup>d</sup>	0.86±0.07 <sup>cd</sup>	1.13±0.28 <sup>bc</sup>	1.25±0.37 <sup>abc</sup>	1.57±0.30 <sup>a</sup>	1.31±0.11 <sup>bc</sup>	9.85
SGR (%)	2.65±0.06 <sup>d</sup>	2.81±0.09 <sup>c</sup>	2.92±0.09 <sup>bc</sup>	3.02±0.09 <sup>ab</sup>	3.06±0.17 <sup>ab</sup>	3.17±0.07 <sup>a</sup>	3.09±0.10 <sup>ab</sup>	8.70

Initial morphometric data: 1.1±0.05cm length; 0.05±0.006 g weight.

Each value is mean ± SD 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.

**Table 2** Activities of digestive enzymes in *M. rosenbergii* PL fed with pelletized artificial feed, un-enriched *Artemia* nauplii and *E. durans* enriched *Artemia* nauplii for 45-days

Enzyme (U/mg protein)	Pelletized Artificial feed fed PL. Jain et al., [39, 40]	Un-enriched <i>Artemia</i> nauplii fed PL. Jain et al., [39, 40]	PL fed with different concentrations of <i>E. durans</i> enriched <i>Artemia</i> nauplii					F-value
			3874×10 <sup>-1</sup> CFU	2671×10 <sup>-3</sup> CFU	1864×10 <sup>-5</sup> CFU	1071×10 <sup>-7</sup> CFU	684×10 <sup>-9</sup> CFU	
Protease	1.44±0.04 <sup>f</sup>	1.53±0.07 <sup>e</sup>	1.78± 0.06 <sup>d</sup>	1.85±0.04 <sup>d</sup>	2.10±0.08 <sup>c</sup>	2.52±0.09 <sup>a</sup>	2.21±0.11 <sup>b</sup>	17.85
Amylase	0.62±0.08 <sup>e</sup>	0.74±0.05 <sup>f</sup>	0.82±0.04 <sup>d</sup>	1.01±0.08 <sup>c</sup>	1.11±0.07 <sup>bc</sup>	1.42±0.1 <sup>a</sup>	1.21±0.08 <sup>b</sup>	40.98
Lipase*	0.12±0.03 <sup>f</sup>	0.18±0.05 <sup>e</sup>	0.20±0.07 <sup>d</sup>	0.40±0.05 <sup>c</sup>	0.45±0.05 <sup>b</sup>	0.59±0.07 <sup>a</sup>	0.49±0.09 <sup>ab</sup>	31.44

Each value is mean ± standard deviation of three individual observations.

\*, unit×10<sup>3</sup>.

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 3** Concentrations of basic biochemical constituents in *M. rosenbergii* PL fed with pelletized artificial feed, un-enriched *Artemia* nauplii and *E. durans* enriched *Artemia* nauplii for 45-days.

Parameter (mg/g wet wt.)	Pelletized Artificial feed fed PL. Jain et al., [39, 40]	Un-enriched <i>Artemia</i> nauplii fed PL. Jain et al., [39, 40]	PL fed with different concentrations of <i>E. durans</i> enriched <i>Artemia</i> nauplii					F-value
			3874×10 <sup>-1</sup> CFU	2671×10 <sup>-3</sup> CFU	1864×10 <sup>-5</sup> CFU	1071×10 <sup>-7</sup> CFU	684×10 <sup>-9</sup> CFU	
Protein	38.64±0.64 <sup>e</sup>	45.44±115 <sup>e</sup>	58.88±1.37 <sup>d</sup>	60.16±0.82 <sup>d</sup>	65.63±1.29 <sup>c</sup>	103.2±1.5 <sup>a</sup>	80.67±1.29 <sup>b</sup>	993.93
Amino acid	21.69±1.68 <sup>e</sup>	24.00±1.19 <sup>d</sup>	30.95±1.05 <sup>d</sup>	42.26±2.60 <sup>c</sup>	49.40±0.71 <sup>b</sup>	62.63±1.21 <sup>a</sup>	51.46±1.60 <sup>b</sup>	295.42
Carbohydrate	12.28±0.72 <sup>d</sup>	14.69±1.50 <sup>e</sup>	17.27±0.62 <sup>d</sup>	20.13±1.43 <sup>c</sup>	21.60±0.66 <sup>c</sup>	29.03±1.80 <sup>a</sup>	24.08±0.99 <sup>b</sup>	69.38
Lipid	5.18±0.24 <sup>d</sup>	6.92±0.36 <sup>e</sup>	8.22±0.18 <sup>e</sup>	10.14±0.24 <sup>d</sup>	11.67±0.07 <sup>c</sup>	15.14±0.37 <sup>a</sup>	12.58±0.21 <sup>b</sup>	541.21

Each value is mean ± standard deviation of three individual observations.  
\*, unit×10<sup>3</sup>.

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).

Similar increases in total protein, amino acid, carbohydrate and lipid levels have been reported by Seenivasan et al., [52] and Jain et al., [39, 40] in *M. rosenbergii* PL fed with *L. sporogenes*, *E. gallinarum* and *E. hirae* enriched *Artemia* nauplii. Increased contents of these basic biochemical constituents have also been recorded in *M. rosenbergii* PL fed with many probiotics supplemented diets [21, 22, 25-30, 59, 60, 70].

### 3.4. Analysis of gut microbial consortium

We have previously identified the presence of *Escherichia coli*, *Klebsiella* sp, *Citrobacter* sp., *Acetobacter* sp., *Streptococcus* sp., *Bacillus* sp., *Staphylococcus* sp., and *Pseudomonas* sp., in the un-enriched *Artemia* nauplii fed control prawn gut through colony morphology and biochemical tests [39, 40], when a parallel experimental set-up was maintained to study the probiotic effects of *E. gallinarum*, *E. hirae* and *E. durans*. In the present study, in the gut of experimental of PL fed with  $1071 \times 10^{-7}$  concentration of *E. durans* enriched *Artemia*, the presence of *Enterococcus* sp., *E. coli*, *Bacillus* sp., and *Klebsiella* sp., were identified through colony morphology and biochemical tests (Tables 4 and 5; Figure 4). Therefore, the pathogenic bacteria, *Citrobacter* sp., *Acinetobacter* sp., *Streptococcus* sp., *Staphylococcus* sp., and *Pseudomonas* sp., were competitively excluded due to the colony establishment of *Enterococcus* sp.

Aquatic animals, particularly at larval stage are monogastric have difficulty in digesting complex or fibrous feedstuffs de facto, thus bacteria colonized in the gut enhanced secretion of digestive enzymes to expedite the digestion [71]. Actually, probiotics have the ability to modulated gut microbiota and subsequently improve digestive enzymes secretion which in turn improves digestion and feed utilization [20, 62, 72, 73]. Probiotics are excellent source of growth promoter and provide vast nutritional benefits, among them the genera of *Bacillus*, could biosynthesize a wide range of extracellular enzymes, such as protease, lipase, amylase and cellulase, and other growth factors, such as biotin, vitamin B<sub>12</sub>, short-chain fatty acids, and essential amino acids [74, 75].

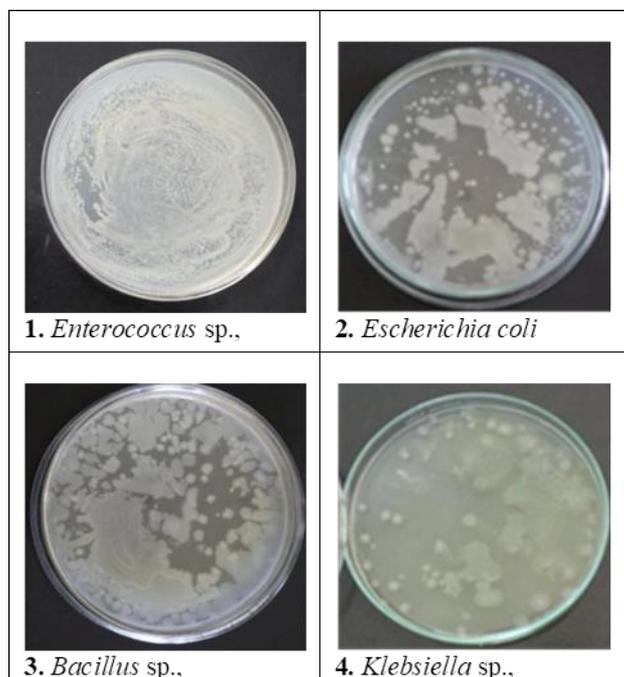
**Table 4** Confirmative results of biochemical tests for micro flora present in the gut of *M. rosenbergii* PL fed with  $1071 \times 10^{-7}$  CFU of *E. durans* enriched *Artemia* nauplii

Test	Gut of PL fed with un-enriched <i>Artemia</i> nauplii Jain et al., [39, 40]									Gut of PL fed with <i>E. durans</i> enriched <i>Artemia</i> nauplii			
	<i>Ec</i>	<i>K</i> sp.,	<i>C</i> sp.,	<i>A</i> sp.,	<i>Ste</i> sp.,	<i>B</i> sp.,	<i>Sta</i> sp.,	<i>P</i> sp.,	<i>E</i> sp.,	<i>Ec</i>	<i>B</i> sp.,	<i>K</i> sp.,	
Gram's staining	-	-	+	-	+	+	+	+	-	+	+	+	-
Motility test	+	+	+	+	+	+	+	+	+	-	+	-	-
Indole test	+	+	+	-	+	-	-	-	-	-	+	-	-
Methyl red test	+	+	+	+	+	-	-	-	-	-	-	+	+
Vp 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	A	+	+	A	A	A	A	A	A	NA	A
Lactose test	A	A	A	+	+	A	A	A	NA	A	A	A	A
Sucrose test	A	A	A	+	-	A	A	A	A	A	A	A	A
Manitol test	A	A	A	+	-	A	A	A	A	NA	NA	A	A
Maltose test	NA	NA	A	+	-	NA	A	NA	A	A	-	+	-

+, Positive; -, Negative; A, Acid production; NA, No acid production. *Ec*, *Escherichia coli*; *K* sp., *Klebsiella* sp.; *C* sp., *Citrobacter* sp.; *A* sp., *Acetobacter* sp.; *Ste* sp., *Streptococcus* sp.; *B* sp., *Bacillus* sp.; *Sta* sp., *Staphylococcus* sp.; *P* sp., *Pseudomonas* sp.; *E* sp., *Enterococcus* sp.,

**Table 5** Bacterial consortium in the gut of *M. rosenbergii* PL fed with un-enriched and  $1071 \times 10^{-7}$  CFU *E. durans* enriched *Artemia* nauplii

Samples	Identified species	Composition (%)
Gut of PL fed with un-enriched <i>Artemia</i> nauplii. Jain et al., [1, 2]	<i>E. coli</i>	10
	<i>Klebsiella</i> sp.,	8
	<i>Citrobacter</i> sp.,	15
	<i>Acinetobacter</i> sp.,	12
	<i>Streptococcus</i> sp.,	11
	<i>Bacillus</i> sp.,	18
	<i>Staphylococcus</i> sp.,	10
	<i>Pseudomonas</i> sp.,	11
	Total	93
	<i>Enterococcus</i> sp.,	32
Gut of PL fed with <i>E. durans</i> enriched <i>Artemia</i> nauplii	<i>E. coli</i>	23
	<i>Bacillus</i> sp.,	23
	<i>Klebsiella</i> sp.,	9
	Total	87

**Figure 4** Agar plate morphology of different bacteria cultured from the gut of *M. rosenbergii* PL fed with *E. durans* (CFU  $1071 \times 10^{-7}$ ) enriched *Artemia* nauplii

It is well known that the microbiota in the gut of aquatic animals can be modified by ingestion of other microorganisms, and therefore, microbial manipulation constitutes a viable tool to eliminate the incidence of opportunist pathogens by maintaining bacterial antagonism (Balcazar, 2002). Competitive exclusion of potential pathogenic bacteria effectively reduces or eliminates the need for antibiotic prophylaxis. For example, *Vibrio alginolyticus* has been used to increase survival and growth of *L. vannamei* PL [76]. *Bacillus* sp., and *E. faecalis* improved growth performance in *L. vannamei* [77]. The lactic acid bacteria enhance the crude protein and ash content in juveniles of *P. indicus* [78].

*Enterococcus lactis* isolated from shrimp samples of *P. vannamei* possesses antagonistic activity against pathogenic bacteria such as *Listeria monocytogenes*, *Pseudomonas aeruginosa*, *Lactococcus garvieae* and against fungi (*Aspergillus niger* and *Fusarium equiseti*). The bacteriocin produced by *E. lactis* was thermostable, active in the pH range from 4.0 to 9.0, has a bactericidal mode of action, showed lack of significant antibiotic resistance genes, virulence determinants and absence of haemolytic activity, and thus recommended to be used as a natural additive or adjunct protective culture in food biopreservation and/or probiotic [79]. It has been reported that *E. durans* and *L. lactis* have in vitro suppression of growth of *Escherichia coli*, *Staphylococcus aureus* and *Shigella flexneri* and therefore, they emerged as new probiotics [80].

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#### 4. Conclusion

The present study concluded that the probiotic bacterium, *E. durans* has got colonized in the gut of *M. rosenbergii*, eliminated some pathogenic bacteria *Citrobacter* sp., *Acinetobacter* sp., *Streptococcus* sp., *Staphylococcus* sp., and *Pseudomonas* sp., and also enhanced the survival and growth. Furthermore, *E. durans* increased activities of digestive enzymes which in turn improve digestibility and absorption of nutrients in *M. rosenbergii*, which ultimately improved the contents of basic biochemical constituents. Therefore, it can be taken as a probiotics to maintain sustainability in prawn culture.

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#### Compliance with ethical standards

##### Acknowledgments

The first author is gratefully acknowledging Bharathiar University, Coimbatore, India for providing research facility.

##### Disclosure of conflict of interest

There is no conflict of interest.

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### How to cite this article

Jain IB, Bhavan PS, Dharani C, Manjula T, Kalpana R, Muralisankar T, Karthik M and Puvitha P. (2020). Growth and survival promotion of a probiotic bacterium *Enterococcus durans* enriched *Artemia* nauplii on the prawn *Macrobrachium rosenbergii*. *GSC Biological and Pharmaceutical Sciences*, 12(1), 87-101.

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