International Journal of Zoological Investigations

Contents available at Journals Home Page: www.ijzi.net

ISSN: 2454-3055

Growth Performance of the Prawn *Macrobrachium rosenbergii* Post-Larvae Fed with Probiotic Bacterium, *Enterococcus hirae* Enriched *Artemia franciscana* Nauplii

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Received: 4th March, 2020
Accepted: 9th April, 2020
Published online: 30th April, 2020

https://doi.org/10.33745/ijzi.2020.v06i01.009

Abstract: This study was aimed to investigate whether the bacterium *Enterococcus hirae* can be used as a probiotic for promoting the growth of the prawn, *Macrobrachium rosenbergii* post-larvae (PL). *Artemia franciscana* nauplii were enriched with *E. hirae* at five different serially diluted concentrations (10^{-1}, 10^{-3}, 10^{-5}, 10^{-7} and 10^{-9}). A 45 day feeding trial revealed that the survival, growth, concentrations of total protein, amino acid, carbohydrate and lipid, and activities of protease, amylase and lipase were significantly ($P<0.05$) increased in experimental PL, particularly at 1764×10^{-7}, CFU. Further, the consortium of the gut microflora of un-enriched *Artemia* nauplii fed PL showed the presence of *Escherichia coli*, *Klebsiella* sp., *Citrobacter* sp., *Acinetobacter* sp., *Streptococcus* sp., *Bacillus* sp., *Staphylococcus* sp. and *Pseduomonas* sp. In *E. hirae* enriched *Artemia* nauplii fed PL showed the presence of *Enterococcus* sp., *E. coli*, *Lactobacillus* sp., *Acinetobacter* sp. and *Staphylococcus* sp. It was found that the pathogenic bacteria *Klebsiella* sp., *Citrobacter* sp., *Streptococcus* sp. and *Pseduomonas* sp. were competitively excluded by the colony establishment of *Enterococcus* sp. in experimental PL. Therefore, *E. hirae* can be used as a probiotic for promoting the sustainable culture of *M. rosenbergii.*

Keywords: *Enterococcus hirae*, *Artemia* nauplii, Prawn, Growth, Survival, Protein, Digestive enzymes, *Macrobrachium rosenbergii*

Citation: Jain I. B., Saravana Bhavan P., Manjula T., Dharani C., Kalpana R., Muralisankar T., Aiswaryalakshmi S. and Karthik M.: Growth performance of the prawn *Macrobrachium rosenbergii* post-larvae fed with probiotic bacterium, E*nterococcus hirae* enriched *Artemia franciscana* nauplii.. Intern. J. Zool. Invest. 6 (1): 107-121, 2020. **https://doi.org/10.33745/ijzi.2020.v06i01.009**

Introduction

Aquaculture practices of the giant river prawn, *Macrobrachium rosenbergii* is profitable one and this species grows fast in either monoculture or poly-culture with major

carps worldwide (FAO, 2016). Probiotics are generally supplemented either with feed or additionally given into the culture environment for enhancing the immune response of cultured organisms. They fight against pathogens by impeding adherence in the intestinal mucosa, releasing the gut protective metabolites including antimicrobial substances, bacterial enzymes such as bacteriocins, siderophores, lysozymes and hydrogen peroxides and thereby competitively excluded the pathogenic bacteria (Farzanfar, 2006; Zai *et al.*, 2009; Ninawe and Selvin, 2009; Barman *et al.*, 2013). Probiotics used as an alternative against antibiotics to boost up the immunoprophylactic measures of diseases by colonizing in the gut, altering microbial composition and maintaining the barrier function (Verschuere *et al.*, 2000; Apines-Amar and Amar, 2015; Chen *et al.*, 2017; Mathipa, 2017).

Enterococcus hirae, *Enterococcus casseliflavus and Enterococcus durans* are frequently occurring components of the intestinal flora of several domestic animal species (Devriese *et al.*, 1987; Ahmed and Baptiste, 2018). Though these are not a part of the normal microbiota in seafood organisms (Boss *et al.*, 2016), the *Enterococcus* genus were isolated from the intestine of common carp and freshwater prawn *M. rosenbergii* (Cai *et al.*, 1999). However, *Enterococci* obtained from food and human samples show virulence characters (Kurekci *et al.*, 2016).

Probiotic feeding treatment for assessment of growth and other physiological and biochemical parameters have widely been reported in aquatic animals; effect of *Lactobacillus* bacteria on growth performance and digestive enzyme activities in the gilthead sea bream *Sparus aurata* (Suzer *et al.*, 2008); *Lactobacillus acidophilus* tested for the better growth performance, haematological parameters and concentration of immunoglobulin in African catfish *Clarias gariepinus* (Al-Dohail *et al.*, 2009); *Bacillus* strains *(Bacillus pumilus* and *Bacillus clausii)* with antagonistic activity to improve the growth performance and immune responses of *Epinephelus coioides* (Sun *et al.*, 2010); *Bacillus subtilis, Bacillus licheniformis, Lactobacillus* sp., *Arthrobacter* sp. and *Vibrio harveyi* tested for the growth, non-specific immunity and disease resistance in cobia *Rachycentron canadum* (Geng *et al.*, 2012); *Bacillus* sp., *Rhodobacter* sp., *Streptococcus* sp., for growth and production of the giant freshwater prawn *M. rosenbergii* (DeMan, 1879) (Ghosh *et al.*, 2016); *Bacillus subtilis* used as dietary supplement to enhance the growth performance and disease resistance against *Vibrio alginolyticus* in parrot fish *Oplegnathus fasciatus* (Liu *et al.*, 2018) and *Bacillus subtilis* and *Bacillus licheniformis* for better growth, feed efficiency, body composition and immune parameters in whiteleg shrimp *Litopenaeus vannamei* (Madani *et al.*, 2018). In this study, *Artemia franciscana* nauplii was enriched with *Enterococcus hirae* and fed to *M. rosenbergii* PL as a live feed for assessing its survival, growth, concentrations of total protein, amino acid, carbohydrate and lipid, and activities of protease, lipase and amylase.

Materials and Methods

Procurement of Enterococcus hirae (3612) and its Sub Culture:

The lyophilized powder of *E. hirae* (MTCC 3612) was procured 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^{-1}), Beef extract (1.5 g L^{-1}), Sodium chloride (5.0 g L^{-1}), and *Yeast* extract (1.5 g L^{-1}). 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 min. A loop of *E. hirae* was inoculated into the broth and incubated for 24 hours at 37 C. The appearance of turbid broth indicates the growth of *E. hirae* (Fig. 1). The cultured *E. hirae* 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 (30 µl) was spread over the agar plate and the appearance of white colony indicates the growth of *E. hirae* (Fig. 2). 20 µl of serially diluted broth (up to 10^{-9}) was spread on nutrient agar for enumerating the CFU in order to optimize it, and the count was 4865 at 10^{-1}, 3264 at 10^{-3}, 2274 at 10^{-5}, 1764 at 10^{-7} and 1071 at 10^{-9}.

Fig. 1: Mother culture morphology of *E. hirae.*

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 methodology (1995) (Table 1).

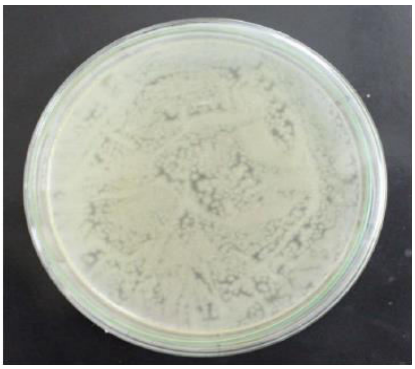

Fig. 2: Spread plate culture morphology of *E. hirae* on nutrient agar.

Enrichment of Artemia nauplii with E. hirae:

The brine shrimp, *A. franciscana* cyst was purchased from Aqua World, Paris Corner, Chennai, India. The cysts (2 g/ 20 L and 15 g kg^{-1} body biomass of the prawns) were taken and hydrated in 1 L^{-1} of purified artificial saltwater (prepared from artificial sea salt powder 35.0 g L^{-1}, pH of 6.5) for 12-15 h. The cysts burst and the embryo surrounded by the

Table 1: Proximate compositions and mineral contents in the basal diet formulated

Proximate composition	(%)
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
Minerals	
Sand and silica (Acid insoluble ash)	0.88
Calcium	0.80
Phosphorus	0.90
Iron	0.11
Copper	0.002
Salt	0.58

hatching membrane become visible for few hours. The brownish orange coloured nauplii came out. The 48 h old *Artemia* nauplii were filtered and transferred to 1 L capacity glass beaker. Five such groups were enriched with 4865×10^{-1}, 3264×10^{-3}, 2274×10^{-5}, $1764\times10^{-7}$and 1071×10^{-9} concentrations of *E. hirae* for 1 h. The *Artemia* nauplii were washed with freshwater and fed to *M. rosenbergii* PL.

Procurement and Acclimatization of M. rosenbergii PL:

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. During acclimatization the prawns were fed with *Artemia* nauplii, boiled egg albumin threads and artificial 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/moults, and dead prawns if any were routinely removed by siphoning without disturbing the prawns while renewing the water medium.

Feeding Trail:

The feeding trials were conducted for a period of 45 days. Seven group of *M. rosenbergii* (1.1±0.05 cm; 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. hirae* (4865×10^{-1}, 3264×10^{-3}, 2274×10^{-5},1764×10^{-7}, 1071×10^{-9}) enriched *Artemia* nauplii. Each group comprised of 30 individuals accommodated in 25 L 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.

Calculation of Nutritional Indices:

The survival rate (SR), length gain (LG), weight gain (WG), and specific growth rate (SGR) were individually calculated (Tekinay and Davis, 2001).

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 W2 – log W1/ t × 100

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

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 (Lowry *et al.*, 1951; Moore and Stein, 1948; Roe, 1955), respectively. The total lipid was extracted gravimetrically (Folch *et al.*, 1957) and spectrophotometrically estimated (Barnes and Blackstock, 1973). The contents of ash and moisture were analysed (AOAC, 1995).

Assays of Digestive Enzymes Activities:

Activities protease, amylase and lipase were assayed on 45th day of feeding trial. The digestive tract of prawns 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 served as crude enzyme source. Total protease activity was determined by casein-hydrolysis method (Furne *et al.*, 2005), where one unit of enzyme activity represented the amount of enzyme required to liberate 1 µg of tyrosine per min. The specific activity of amylase was calculated as mg of maltose liberated per g of starch per hour (Bernfeld, 1955). Lipase activity was assayed and calculated as the amount of free fatty acid released from triacylglycerol per unit time (Furne *et al.*, 2005).

Gut Microbial Colonization:

The bacterial culture was performed in the gut homogenate of experimental prawns fed with *E. hirae* (1764×10^{-7}). The prawns were deactivated by keeping them in freezer at -20 C for 10 min. 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 (Holt *et al.*, 1996). The bacterial colony was enumerated by using the formula-- Bacterial count (CFU/ g) = Number of colonies × Dilution factor/ Volume of sample (g).

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 (95%) was considered as statistically significant.

Results and Discussion

Survival Rate and Nutritional Indices:

The survival rate (SR) and growth rate (WG and SGR) were found to be significantly ($P<0.05$) higher at all concentrations (4865×10^{-1}, 3264×10^{-3}, 2274×10^{-5}, 1764×10^{-7}, and 1071×10^{-9}) of *E. hirae* enriched *Artemia* nauplii fed PL when compared with un-enriched *Artemia* and the PL fed with pelletized feed. Among these concentrations,

1764×10^{-7} has produced the best growth performance (Fig. 3; Table 2).

A **B**

Fig. 3: *A. franciscana* nauplii (24 hrs., old). **A:** Un-enriched; **B:** Enriched with *E. hirae* (CFU=1764×10^{-7}).

The following studies support our result on growth and survival: in Angelfish, *Pterophyllum scalare* fed with *Artemia* nauplii enriched with symbiotic bacterium (*Pediococcus acidilactici* and fructo-oligosaccharide) (Mahmood *et al.,* 2016); in *M. rosenbergii* fed with *Lactobacillus sporogenes* enriched *Artemia*, Binifit™, *Bacillus. coagulans* and *Bacillus subtilis* (Seenivasan *et al.*, 2011, 2012, 2014; Karthik *et al.*, 2018); in shrimp *Litopenaeus vannamei* fed with microencapsulated and freeze-dried *Bacillus* (Nimrat *et al.*, 2012). Ranjit Kumar *et al.*, (2017) reported that *Bacillus licheniformis* significantly reduced the cumulative mortality of *M. rosenbergii* challenged with *Vibrio alginolyticus* due to improved immunity. According to Liu *et al.* (2010), *Bacillu*s sp., administration had increased survival rate of shrimp due to enhanced stress resistance. A significant increase in weight gain was reported in parrot fish fed with the diet containing *Bacillus subtilis* (Liu *et al.*, 2018).

Activities of Digestive Enzymes and Contents of Basic Biochemical Constituents:

Activities of protease, amylase and lipase, and concentrations of total protein, amino acids, carbohydrate, and lipids were found to be significantly ($P< 0.05$) elevated in *E. hirae* enriched *Artemia* nauplii fed *M. rosenbergii* PL when compared with un-enriched *Artemia* and artificial pelletized feed**.** Among different concentrations of *E. hirae*, 1764×10^{-7} produced the best performance (Table 3).

Bacillus sp. generally use a variety of nutrients for their growth and simultaneously release relevant digestive enzymes and other necessary growth factors that facilitate nutrient assimilation in their hosts resulting in prevention of intestinal disorders and higher growth and survival (Wang, 2007; Sahu *et al.*, 2008; Lara-Flores, 2011). Activities of digestive enzymes of *Penaeus vannamei* larvae were significantly increased when using *Bacillus coagulans* in their feeding regime (Zhou *et al.*, (2009). Some other studies also reported that probiotic bacteria could participate in the digestion processes by producing protease, amylase and lipase, as well as some necessary growth factors in the hosts (Arcllano and Olmos 2002; Wang and Xu 2006; Wang 2007; Motlagh *et al.*, 2012). Some non-probiotic strains, such as genetically modified *Escherichia coli*, were also reported to be able to produce large amount of proteases and lipases, which could facilitate the digestive processes in *Fenneropenaeus indicus* post-larvae (Sirvas-Cornejo *et al.*, 2007). An increased activity of digestive enzymes was reported in *Penaeus vannamei* when *Bacillus coagulans* SC8168 given even at the latter stages (Zhou *et al.*, 2009). Enhanced protease activity was reported in *Litopenaeus*

Table 2: Survival and growth of *M. rosenbergii* PL fed with pellet feed, un-enriched and *E. hirae* enriched *Artemia* nauplii for 45 days.

Parameter	Pelletized feed	PL fed with Un-enriched *Artemia* nauplii	PL fed with *E. hirae* enriched *Artemia* nauplii					F-value
			4865×10^{-1} CFU	3264×10^{-3} CFU	2274×10^{-5} CFU	1764×10^{-7} CFU	1071×10^{-9} CFU	
SR (%)	71.77 ± 1.92^{f}	76.66 ± 3.37^{e}	81.11 ± 5.10^{bc}	85.55 ± 1.92^{ab}	88.88 ± 1.92^{a}	90.00 ± 3.33^{a}	87.77 ± 1.92^{a}	12.82
Length(cm)	1.33 ± 0.06^{g}	1.67 ± 0.06^{f}	1.71 ± 0.09^{d}	1.83 ± 0.07^{d}	2.61 ± 0.10^{b}	3.25 ± 0.14^{a}	2.19 ± 0.21^{c}	94.08
Weight (g)	0.49 ± 0.07^{f}	0.72 ± 0.05^{e}	0.93 ± 0.15^{cd}	1.06 ± 0.14^{bc}	1.30 ± 0.09^{b}	1.44 ± 0.14^{a}	1.14 ± 0.22^{bc}	73.45
LG (cm)	0.33 ± 0.11^{g}	0.66 ± 0.11^{f}	0.71 ± 0.16^{d}	0.83 ± 0.13^{d}	1.61 ± 0.09^{b}	2.25 ± 0.10^{a}	1.19 ± 0.18^{c}	18.24
WG (g)	0.45 ± 0.06^{g}	0.67 ± 0.05^{ef}	0.89 ± 0.15^{cd}	1.02 ± 0.14^{bc}	1.09 ± 0.02^{bc}	1.39 ± 0.14^{a}	1.25 ± 0.09^{ab}	18.54
SGR (%)	2.65 ± 0.06^{e}	2.81 ± 0.09^{cd}	2.93 ± 0.16^{sb}	2.99 ± 0.14^{ab}	3.01 ± 0.15^{ab}	3.12 ± 0.13^{a}	3.08 ± 0.12^{b}	4.77

Initial morphometric data: 1.1±0.05 cm length; 0.05±0.006 g weight.
Each value represents 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 3: Concentrations of biochemical constituents and activities of digestive enzymes in *M. rosenbergii* PL fed with pelletized feed, un-enriched and *E. hirae* enriched *Artemia* nauplii for 45 days.

Parameter		Pelletized feed	PL fed with Un-enriched *Artemia* nauplii	PL fed with *E. hirae* enriched *Artemia* nauplii					F-value
				4865×10^{-1} CFU	3264×10^{-3} CFU	2274×10^{-5} CFU	1764×10^{-7} CFU	1071×10^{-9} CFU	
Biochemical constituents (mg/g wet wt.)	Protein	38.64 ± 0.65^{e}	45.44 ± 1.15^{f}	60.00 ± 0.97^{e}	70.44 ± 0.82^{c}	66.53 ± 1.29^{d}	104.06 ± 1.35^{a}	81.42 ± 1.31^{b}	173.54
	Amino acid	21.69 ± 1.69^{e}	24.00 ± 1.19^{e}	32.14 ± 1.05^{d}	43.52 ± 2.59^{c}	50.53 ± 0.64^{b}	63.82 ± 1.21^{a}	52.64 ± 1.60^{b}	298.27
	Carbo-hydrate	12.28 ± 0.72^{d}	14.69 ± 1.50^{e}	17.77 ± 0.62^{d}	20.29 ± 1.51^{c}	22.06 ± 0.48^{c}	29.39 ± 1.49^{a}	24.69 ± 0.98^{b}	79.80
	Lipid	5.18 ± 0.24^{f}	6.92 ± 0.36^{g}	8.49 ± 0.23^{e}	10.38 ± 0.23^{d}	12.02 ± 0.05^{c}	15.59 ± 0.61^{a}	12.93 ± 0.29^{b}	366.39
Digestive enzymes (U/ mg protein)	Protease	1.44 ± 0.04^{e}	1.53 ± 0.07^{e}	1.82 ± 0.04^{d}	1.92 ± 0.08^{d}	2.22 ± 0.08^{c}	2.62 ± 0.09^{a}	2.34 ± 0.09^{b}	98.38
	Amylase	0.62 ± 0.08^{e}	0.74 ± 0.05^{de}	0.86 ± 0.05^{d}	1.08 ± 0.07^{cd}	1.18 ± 0.1^{c}	1.51 ± 0.09^{a}	1.32 ± 0.09^{b}	52.18
	Lipase*	0.12 ± 0.03^{c}	0.18 ± 0.05^{c}	0.22 ± 0.05^{c}	0.44 ± 0.08^{b}	0.48 ± 0.05^{b}	0.61 ± 0.07^{a}	0.51 ± 0.10^{ab}	25.21

Each value represents mean ± standard deviation of three individual observations. *, unit$\times10^{3}$
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

vannamei fed with *Bacillus subtilis* E20 (Liu *et al.*, 2009).

An increase in total protein, free amino acid, total carbohydrate, total lipids and ash contents in *M. rosenbergii* PL has been reported when *L. sporogenes* incorporated diet was given (Seenivasan *et al.*, 2014). Similarly, Jayanthi *et al.* (2015 a, b) reported that concentrations of total protein, carbohydrate and lipid were increased in Lactobasil®$_{plus}$ incorporated feed fed *M. rosenenbergii* PL. Fernandez *et al.* (2011) reported that Lactic acid bacteria enhanced the crude protein and ash content in juveniles of *Penaeus indicus*.

Probiotics have been reported to increase intestinal enzyme activities and thus improve nutrient digestibility and food absorption in pearl spot *Etropus suratensis* and tilapia *Oreochromis mossambicus* (Sankar *et al.*, 2016) and narrow clawed crayfish *Astacus leptodactylus* Eschscholtz (Valipour *et al.*, 2019). It has been reported that *Lactobacillus pentosus* improved growth performance due to increased activities of digestive enzymes in *Litopenaeus vannamei* (Zhen and Wang, 2016). Addition of *Lactobacillus delbrueckii* as feed additive has significantly improved lipase activity in common carp (Zhang *et al.*, 2018). Probiotics have the ability to modulate gut microbiota and subsequently improve digestive enzymes secretion which in turn improves digestion and feed utilization -- in white shrimp, *Litopenaeus vannamei* by using *Bacillus coagulans* (Wang *et al.*, 2012) and *Bacillus subtilis* (Zokaeifar *et al.*, 2012); using amylolitic bacteria as candidates of probiotics in tilapia, *Oreochromis* sp. (Putra *et al.*, 2015); in snakehead, *Channa argus* using *Bacillus amyloliquefaciens* (Dai *et al.*, 2018). It has been reported that an increased protein, fat and total digestibility in catfish (*Clarias* sp.) positively correlated to final weight, weight gain, PER, and SGR (Afrilasari *et al.*, 2016). Similar results were reported in white shrimp *Litopenaeus vannamei* that *Bacillus subtilis* in its feed has increased crude protein digestibility, crude lipid digestibility and dry matter apparent digestibility (Tsai *et al.*, 2019). The bacteria, live feeds and their extracellular/exogenous enzymes could boost the production of endogenous enzymes (protease, lipase, and cellulase) in animals including fish and shrimp, which further enhance feed utilization and growth performance-- in Japanese sardine, *Sardinops melanotictus* by feeding rotifer (Kurokawa *et al.*, 1998) and wild mixed zooplankton (Manickam *et al.*, 2020); in Indian white shrimp, *Fenneropenaeus indicus* by giving *Bacillus* sp. (Ziaei-Nejad *et al.*, 2006); in *Penaeus vannamei* by giving *Bacillus coagulans* (Zhou *et al.*, 2009).

Analysis of Gut Microbial Consortium:

Aquatic animals are monogastric and have difficulty in digesting complex or fibrous feedstuffs de facto, thus bacteria colonized in the gut enhanced secretion of digestive enzymes in the larval stage to help them to expedite the digestion on the foodstuffs to be more effective at later stages (Ronnestad *et al.*, 2003; Dawood and Khosio, 2016). This mechanism is of particularly important during the weaning stages due to low level of enzyme production in the early life of fishes and prawns. This has been studied in Roach, *Rutilus rutilus*, where presence of *Aeromonas, Pseudomonas, Enterobacteriaceae, Flavobacterium, Micro-coccus and Acinetobacter* were identified (Skrodenyte-Arbaciauskiene, 2007).

The presence of *Escherichia coli*, *Klebsiella* sp., *Citrobacter* sp., *Acetinobacter* sp., *Streptococcus* sp., *Bacillus* sp., *Staphylococcus* sp. and *Pseudomonas* sp. were identified through colony morphology and biochemical tests in the gut of un-enriched *Artemia* fed PL. In the gut of experimental PL fed with 1764×10^{-7} concentration of *E. hirae* enriched *Artemia* produced the presence of *Enterococcus* sp.*, Escherichia coli, Lactobacillus* sp., *Acinetobacter* and *Staphylococcus* sp. which were identified through colony morphology and biochemical tests. Thus, in this study, *Klebsiella* sp., C*itrobacter* sp., *Streptococcus* sp., and *Pseduomonas* sp. were competitively excluded by colony establishment of *Enterococcus* sp. in experimental PL (Figs. 4, 5; Tables 4, 5).

Bacterial antagonism is a common phenomenon in nature; therefore, microbial interactions play a major role in the equilibrium between competing beneficial and potentially pathogenic microorganisms. However, the composition of microbial communities can be altered by husbandry practices and environmental conditions that stimulate the proliferation of selected bacterial species. It is well known that the microbiota in the gastrointestinal tract of aquatic animals can be modified, for example by ingestion of other microorganisms; therefore, microbial manipulation constitutes a viable tool to reduce or eliminate the incidence of opportunist pathogens (Balcazar, 2002).

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 cellulose and other growth

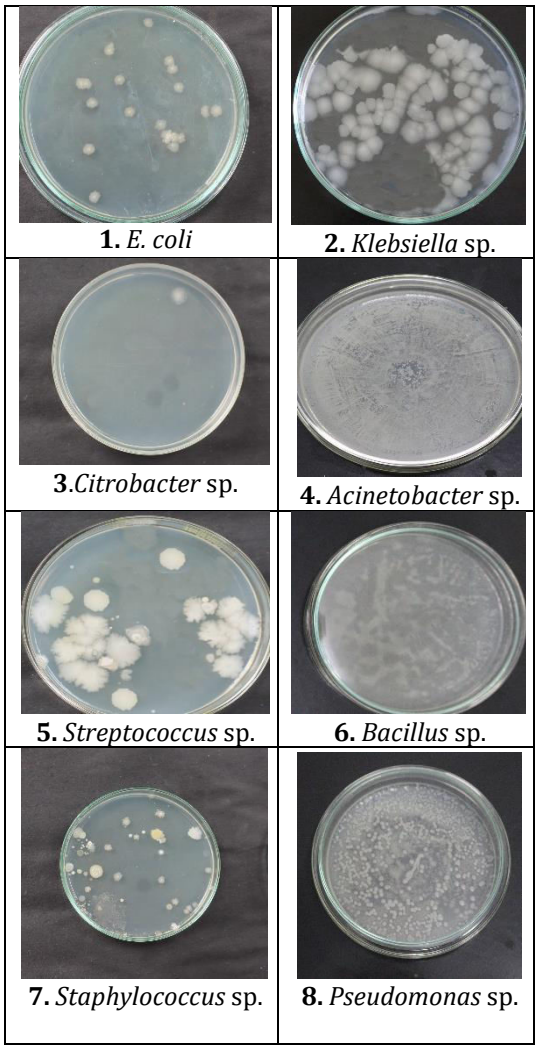

Fig. 4: Agar plate morphology of different bacterial culture from the gut of *M. rosenbergii* PL fed with un-enriched *Artemia* nauplii.

factors such as biotin, vitamin B_{12}, short-chain fatty acids, and essential amino acids. Therefore *Bacillus* sp. enhanced the growth of *Penaeus monodon* when supplemented with diet (De *et al.*, 2018). The usage of *Bacillus* sp. (*B. thuringiensis, B. megaterium, B. polymyxa, B. licheniformis* and *B. subtilis*) and *Yeast* enhanced microbiota, promote survival and growth of *Litopenaeus vannamei* juveniles (Nimrat *et al.*, 2019). *Bacillus* sp. when used as probiotics were able to inhibit pathogens by colonizing both the culture water and the shrimp digestive tract to exclude other harmful bacteria in *Penaeus monodon* and

Table 4: Confirmative results of biochemical tests for micro flora present in the gut of *M. rosenbergii* PL fed with 1764×10^{-7} CFU of *E.hirae* enriched *Artemia* nauplii.

Test	*Eh*	Un-enriched *Artemia* nauplii fed PL gut								*E. hirae* enriched *Artemia* nauplii fed PL gut				
		Ec	*K* sp.	*C* sp.	*A* sp.	*Ste* sp.	*B* sp.	*Sta* sp.	*P* sp.	*E* sp.	*Ec*	L sp.	*A* sp.	*Sta* 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	-	-	+	+	+	-	+	+	+	-	-	+	+	+
Glucose test	A	A	A	A	A	A	A	A	A	A	A	A	A	A
Lactose test	A	A	A	A	NA	NA	NA	A	NA	A	A	A	A	NA
Sucrose test	A	A	A	A	NA	A	A	A	A	A	A	A	A	A
Manitol test	A	A	A	A	NA	A	A	A	A	NA	A	A	A	A
Maltose test	A	NA	A	A	NA	NA	A	NA	A	A	A	A	A	A

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

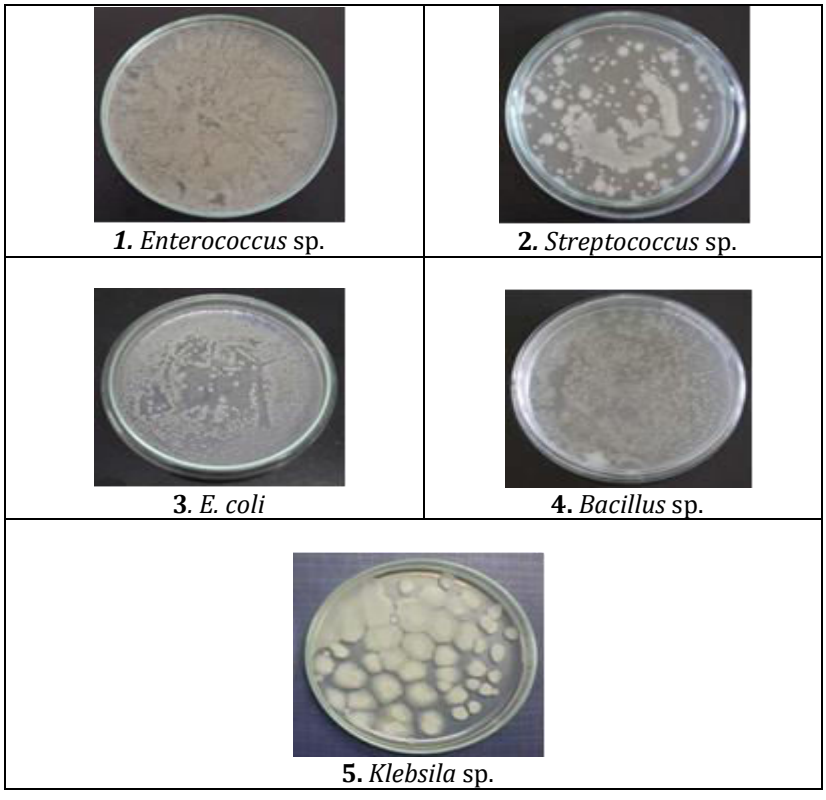

Fig. 5: Agar plate morphology of different bacterial culture from the gut of *M. rosenbergii* PL fed with *E. hirae* (CFU=1764×10^{-7}) enriched *Artemia* nauplii.

Table 5: Bacterial consortium in the gut of *M. rosenbergii* PL fed with un-enriched and 1764×10^{-7} CFU of *E. hirae* enriched *Artemia* nauplii.

Samples	Identified species	Composition (%)
Un-enriched *Artemia* nauplii fed PL gut	*E. coli*	10
	Klebsiella sp.,	8
	Citrobacter sp.,	15
	Acinetobacter sp.,	12
	Streptococcus sp.,	11
	Bacillus sp.,	18
	Staphylococcus sp.,	10
	Pseduomonas sp.,	11
	Total	**93**
E. hirae enriched *Artemia* nauplii fed PL gut	*Enterococcus* sp.,	34
	E. coli	21
	Lactobacillus sp.,	18
	Acinetobacter sp.,	8
	Staphylococcus sp.,	7
	Total	**88**

Penaeus vannamei, producing an anti-bacterial substance or activating both cellar and humoral immune defences in shrimp (Rengpipat *et al.*, 1998; Mariel *et al.*, 2004). *Bacillus subtilis and Bacillus licheniformis* improved survival, growth and feed utilization efficiency of *Litopenaeus vannamei* after feeding with diets (Madani *et al.*, 2018). *Bacillus* sp., and *Enterococcus faecalis* improved growth performance in *Litopenaeus vannamei* (Guzmán-Villanueva *et al.*, 2019). *Bacillus subtilis* activating the immunity of white shrimp, *Litopenaeus vannamei* and improves shrimp health (Chien *et al.*, 2020). Supplementation of *Bacillus subtilis* has improved the growth performance, immune response and antioxidant activities in *Litopenaeus vannamei* (Shen *et al.*, 2010). Thus, *Bacillus* spp. have served as promising probiotics, able to inhibit colonization of prospective pathogens in the intestine of shrimp, thereby boosting shrimp immunity and disease resistance. Competitive exclusion of potential pathogenic bacteria effectively reduces or eliminates the need for antibiotic prophylaxis. For example, *Vibrio alginolyticus* has also been used to increase survival and growth of *Litopenaeus vannamei* PL (Garriques and Arevalo, 1995).

Conclusion

In this study, *E. hirae* got colonized in the gut of *M. rosenbergii,* eliminated *Klebsiella* sp., C*itrobacter* sp., *Streptococcus* sp. and *Pseduomonas* sp. and enhanced the survival and growth of the host. Therefore, it can be taken as a probiotic bacterium to maintain sustainability in prawn culture.

Acknowledgement

The authors gratefully acknowledge Bharathiar University, Coimbatore, India for providing laboratory facility.

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