ISSN: 0975-833X

Available online at http://www.journalcra.com

International Journal of Current Research
Vol. 7, Issue, 01, pp.11440-11453, January, 2015

INTERNATIONAL JOURNAL OF CURRENT RESEARCH

RESEARCH ARTICLE

PROBIOTICS PRODUCT (LactoBacil®$_{plus}$) ON IMPROVEMENT OF SURVIVAL, GROWTH, DIGESTIVE ENZYMES ACTIVITY, NUTRITIONAL STATUS AND GUT MICROFLORA OF THE PRAWN *MACROBRACHIUM ROSENBERGII*

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ARTICLE INFO

Article History:
Received 20th October, 2014
Received in revised form
18th November, 2014
Accepted 14th December, 2014
Published online 23rd January, 2015

Key words:

Macrobrachium rosenbergii,
LactoBacil®$_{plus}$,
Survival,
Growth,
Protease,
Amylase,
Lipase,
Protein,
Amino Acids,
Fatty Acids,
Gut microbes,
16S rDNA.

ABSTRACT

In the present study, commercially available probiotics product, LactoBacil®$_{plus}$ (LBP), which contains a combination of *Lactobacillus acidophilus, Lactobacillus rhamnosus, Bifidobacterium longum, Bifidobacterium bifidum* and *Saccharomyces boulardii* was incorporated at 1, 2, 3, 4 and 5% concentrations with basal diet formulated with fish meal, groundnut oil cake, soya bean meal, corn flour, tapioca flour, egg albumin, Cod liver oil and vitamin B-complex with vitamin-C, and fed to *Macrobrachium rosenbergii* PL for 90 days. The beneficial effects of LBP on the survival, growth, nutritional indices (weight gain, specific growth rate, feed conversion ratio, and protein efficiency ratio), activities of digestive enzymes (protease, amylase and lipase), concentrations of biochemical constituents (total protein, carbohydrate and lipid) including profiles of protein, amino acids and fatty acids were determined. In addition to these, gut microbial colony establishment and their biochemical characterization, and the molecular analysis of gut bacterial diversity through 16s rDNA were also done. The survival, growth, nutritional indices, activities of protease, amylase and lipase, concentrations of total protein, carbohydrate and lipid, level of essential amino acids and fatty acids (linoleic and ecosapentanoic acid) were found to be significantly ($P<0.05$) improved particularly at 4% LBP incorporated diet fed prawns when compared with control. The LBP incorporated feed fed prawn showed increased staining intensity of 36, 29 and 18 kDa protein bands when compared with control. Presence of *Bacillus* spp., *Pseudomonas* spp., *Escherichia coli,* and *Streptococcus* spp., was deducted in the gut of control prawns. In the gut of experimental prawns in addition to *L. acidophilus, L. rhamnosus, B. longum, B. bifidum* and *S. boulardii* the presence of *E. coli* was also deducted. However no *Pseudomonas* sp., was deducted in the gut of experimental prawns. The bacterial consortium of control and experimental prawn guts showed 98% and 93% similarity respectively with *Ralstonia* (genes of proteobacterium). This study revealed that the identified gut microbes were differed from originally incorporated probiotics and unidentified bacterial species was present. This needs further clarification. In conclusion, the LBP at 4% was found to be beneficial for survival, growth and production of *M. rosenbergii*. Therefore, this can be recommended to incorporate in aqua feed formulations for the sustainable development of *Macrobrachium* culture.

INTRODUCTION

Aquaculture is one of the fastest growing food production sectors. It plays a vital role in offering better and quality nutrition (protein, amino acids, fatty acids, minerals, vitamins and fibre), employment generation and rural development. FAO (2012) reported that the world production of marine and freshwater crustaceans contributes 17.3% and 4.5% respectively. Among the freshwater prawns, *Macrobrachium malcolmsonii, Macrobrachium gangeticum, Macrobrachium amazonicum* and *Macrobrachium rosenbergii* have commercial importance. Farming of the latter giant river prawn, *M. rosenbergii* has been gained increased interest in recent years due to its high economic value in the world market (Radheyshyam, 2009). The top four producers of this species in 2007 were China, Thailand, India and Bangladesh (FAO, 2009). The annual production of over 30,000 tonnes has been achieved for *M. rosenbergii* through monoculture practices (FAO, 2010).

Probiotics are cultured products or live microbial feed supplements, which are defined as live microorganisms, conferring a health benefit on the host when being consumed in right quantity (FAO/WHO, 2001). In recent years, probiotics have been increasingly used in the biological control to prevent diseases in aquaculture, which will make aquaculture products more environmental friendly and acceptable to consumers (Gatesoupe, 1999; Verschuere *et al*., 2000). Actually, probiotics improve intestinal microbial balance, thereby

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increase activities of digestive enzymes and the food absorption, and reduces pathogenic load in the gastrointestinal tract by competitive exclusion (Holzapfel *et al.,* 1998; Rengpipat *et al.,* 1998; Uma *et al.,* 1999; Gomez-Gill *et al.,* 2000; Balcazar *et al*., 2006). The beneficial effects of many probiotics have been reported on prawns: *Lactobacillus* spp., and *Streptococcus* spp., improved the growth and survival of the green tiger shrimp, *Penaeus semisulcatus* when supplemented with clam meat and pellet feed, Irawan-300-Grower (Murugesan *et al.,* 2008); *Bacillus* spp., improved the digestive enzyme activity and the growth of *Litopenaeus vannamei* (Gomez *et al*., 2008); *Bacillus* spp., improved the water quality and production of *Penaeus vannamei* in ponds (Wang *et al.,* 2005); *Bacillus* S11 increased the survival and growth of *Penaeus monodon* when fed with feed (Rengpipat *et al.,* 2000); *Lactobacillus plantarum* induced the immune modulation, enhanced the immune ability and increased its resistance to *Vibrio alginolyticus* infection in *L. vannamei* (Chiu *et al*., 2007) when fed with diet; *Saccharomyces boulardii* profound beneficial effect on the nauplii of *Artemia* by increasing its resistance to a pathogenic *Vibrio* infection (Patra and Mohamed, 2003); *Phaffia rhodozyma* increased the biomass and survival rate in *P. vannamei* juveniles (Scholz *et al.,* 1999); Binifit™, *Lactobacillus sporogenes*, *Bacillus subtilis* and *Saccharomyces cerevisiae* showed better survival, growth, biochemical constituents and energy utilization in PL of *M. rosenbergii* when supplemented with feed (Seenivasan *et al*., 2011, 2012a-d, 2014a-c).

In the present study, commercially available probiotics product LactoBacil®plus (LBP), which contains a combination of *Lactobacillus acidophilus, Lactobacillus rhamnosus, Bifidobacterium longum, Bifidobacterium bifidum* and *Saccharomyces boulardii* was incorporated with basal diet and fed to *M. rosenbergii* PL for determining its beneficial effects on the survival, growth, nutritional indices (weight gain, specific growth rate, feed conversion ratio, and protein efficiency ratio), activities of digestive enzymes (protease, amylase and lipase), concentrations of biochemical constituents (total protein, carbohydrate and lipid) including profiles of protein, amino acids and fatty acids. In addition to these, biochemical characterization of gut microbial population for understanding the colony establishment, and the molecular characterization of gut bacterial consortium through 16S rDNA analysis to see its genetic diversity were also done.

MATERIALS AND METHODS

Procurement and acclimatization of *M. rosenbergii* PL

The post larvae (PL-5) of *M. rosenbergii* were procured from Aqua Hatcheries, Happy Bay Annexe, Mugaiyur Village, East Coast Road, Cheyyur Taluk, Kanchipuram (Dt), Tamilnadu, India. They were transported to the laboratory in polythene bags filled with oxygenated water. The prawns were acclimatized to the ambient laboratory condition with ground water in cement tanks (6×3×3 feet) for 2 weeks. The ground water satisfied the required physico-chemical parameters (Temperature, 28 ± 2.0°C; pH, 7.0 ± 0.10; total dissolved solids, 0.94 ± 0.08 g L^{-1}; dissolved oxygen, 7.20 ± 0.20 mg L^{-1}; BOD, 34.0 ± 2.0 mg L^{-1}; COD, 130.0 ± 10.0 mg L^{-1}; ammonia, 0.028 ± 0.006 mg L^{-1}). During acclimatization the prawns were fed with boiled egg albumin, live *Artemia* nauplii and commercially available scampi feed. More than 50% of tank water was routinely changed every day in order to maintain a healthy environment and aeration was also provided. This ensures sufficient oxygen supply to the prawns and an environment devoid of accumulated metabolic wastes. The unfed feeds, faeces, moult and dead prawns if any were removed by siphoning without disturbing the prawns.

Purchase of feed ingredients and probiotic product

The feed ingredients, such as fishmeal, soybean meal, groundnut oilcake, corn flour, tapioca flour, cod liver oil and egg were purchased from local market. In addition to this, Vitamin-B complex with vitamin C, and the Indian commercial pre and probiotic product, LactoBacil®plus (LBP) (Organon Ltd, Mumbai, India) were purchased from local medical shop. The LBP contains five different probiotics, *Lactobacillus acidophilus, Lactobacillus rhamnosus, Bifidobacterium longum, Bifidobacterium bifidum* and *Saccharomyces boulardii*.

Feed preparation

The experimental diets were prepared with selected feed ingredients as per ‘‘Pearson’s square-method’’ using pre determined value of 40% protein content, and the compositions of basal diets are given in Table 1.

Table 1. Composition of LBP incorporated experimental diets

Ingredients (g/100g)	Control	LBP incorporated diets				
		1%	2%	3%	4%	5%
Fish meal	21.25	22.25	22.25	24.25	24.25	27.25
Ground nut oil cake	21.25	21.25	20.25	19.25	17.25	17.25
Soya bean meal	21.25	22.25	22.25	22.25	23.25	20.25
Corn flour	11.08	8.08	8.08	6.08	6.08	5.08
Tapioca flour	11.09	11.09	11.09	11.09	11.09	11.09
Egg albumin	11.08	11.08	11.08	11.08	11.08	11.08
Cod liver oil	2.00	2.00	2.00	2.00	2.00	2.00
Vitamin mix*	1.00	1.00	1.00	1.00	1.00	1.00
LBP (%)	0.00	1.00	2.00	3.00	4.00	5.00
Total	100	100	100	100	100	100
8 hr. Leaching (%)	22	23	24	24	23	24

LBP, LactoBacil®plus.

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

Fishmeal, soybean meal and groundnut oilcake were used as protein sources; corn flour and tapioca flour were used as carbohydrate sources; cod liver oil was used as lipid source; tapioca flour and egg albumin were served as binding agents; vitamin B complex with vitamin C was also added. The proportion of each ingredient required was calculated precisely providing allowance for the premix along with tapioca powder and stream cooked then cooled at room temperature (28ºC). Then egg albumin, Cod liver oil, vitamin B-complex with vitamin-C was added one by one. LBP was incorporated with the basal diet at 1%, 2%, 3%, 4% and 5% concentrations. Diet with ‘0’ % incorporation of probiotics product was served as control. Thus six experimental diets were prepared. The dough was prepared for each formulation and pelletized separately.

The pellets were dried in a thermostatic oven (M/s. Modern Industrial, Mumbai, India) at 40ºC until they reached constant weight, and stored in airtight jars at room temperature. In the present study, the proximate composition of organic matters was determined by adopting the methodology of AOAC (1995). Analysis of total nitrogen was performed after single acid digestion (con. H_2SO_4) using Kjeldhal techniques, titrated against 0.1N HCl and the crude protein content was calculated (N*6.25). The crude fat was extracted with petroleum ether, desiccated and weighed. For crude fiber, sample was successively digested by boiling acid and alkali. The extract was converted into ash and the difference was calculated. The sample was ignited in a muffle furnace and the inorganic residue was calculated as total ash. The feed sample was placed in a hot air oven at slightly >100ºC and the loss of weight calculated as the moisture content. The basal diet formulated contains 39.79% crude protein, 6.10% crude fat, 5.43% crude fibre, 8.0% total ash, 7.0% moisture and 33.68% carbohydrate (total nitrogen free extract). The proximate compositions of organic matters present in these formulated diets are presented in Table 2. These diets were freshly prepared once in every 30 days to ensure the maintenance of high probiotic viability throughout the duration of feeding trail. The water stability of the feeds formulated was checked and the leaching percentage after 8 h of immersion was found to be between 22-24% by immersion and drying method.

Table 2. Proximate composition of experimental diets formulated with incorporation of LBP

Composition	Control	LBP incorporated diets				
		1%	2%	3%	4%	5%
Crude Protein	39.79±1.20	40.62±1.5	39.57±1.0	39.86±1.32	39.47±1.02	39.53±1.10
Crude Fat	6.10±0.31	6.09±0.47	6.06±0.54	6.06±0.63	5.91±0.51	6.07±0.45
Crude Fiber	5.43±0.47	4.37±0.38	4.36±0.41	4.18±0.45	4.77±0.56	4.89±0.39
Total Ash	8.0±0.65	9.0±1.17	10.0±1.32	7.0±1.25	8.0±1.20	11.0±1.32
Moisture	7.0±1.01	7.0±1.0	7.0±1.03	8.0±1.0	9.0±1.01	8.0±1.10
Carbohydrate *	33.68±1.23	32.92±1.20	33.01±1.21	34.9±1.0	32.85±1.05	30.51±1.12

Each value is mean ± standard deviation of three individual observations.
LBP, LactoBacil®$_{plus}$; *Total nitrogen free extract.

Experimental procedure

After acclimatization the prawns were transferred to plastic aquaria, where they were allowed to acclimatize the new environment for three days and were devoid of feeding for 24 hr before commencement of the experiment. Approximately PL-30 (1.59 ± 0.33 cm length and 0.064 ± 0.038 g weight) staged prawns were subjected to feeding trail for a period of 90 days. Six groups of 30 prawns each were maintained in 40 L capacity plastic tanks in triplicate. One group served as control and fed with feed devoid of LBP. The experimental groups were fed with the respective concentrations of LBP incorporated feeds two times a day (6:00 am & 6:00 pm) at 10% of body weight. The water (100%) was renewed daily and aerated. The unfed feed, faeces, moult and dead prawns if any were removed by siphoning without disturbing the prawns. The similar experimental set up was maintained then and there to study different parameters.

Analysis of nutritional indices

On the final day of feeding trial the morphometric data, such as the final length and weight were measured for calculating the growth parameters, such as survival rate (SR), weight gain (WG), specific growth rate (SGR), feed conversion ratio (FCR), food conversion efficiency (FCE) and protein efficiency ratio (PER) were calculated by following these equations derived by Tekinay and Davies (2001). Survival rate, SR (%) = Total No. of PL alive at the end of experiment/ Total No. of PL introduced at the beginning of the experiment × 100. Length gain, LG (cm) = The difference between final and initial length (cm) of PL. Weight gain, WG (g) = The difference between final and initial weight (g) of PL. Specific growth rate, SGR (%) = $_{log}$ of final weight (g) − $_{log}$ of initial weight (g)/ No. of experimental days × 100. Feed conversion ratio, FCR (g) = Food consumed (g)/ WG (g). Protein efficiency ratio, PER (g) = WG (g)/ Protein intake (g). For morphometric data 3 sets of 5 prawns in each group were measured individually and the mean of 5 prawns was considered as a single observation and 3 such observations were made.

Assays of digestive enzymes activities

Activities of digestive enzymes, such as protease, amylase and lipase were assayed on initial and final days of feeding trial. The digestive tract of prawn was carefully dissected out and homogenized in ice-cold distilled water and centrifuged at 9329g under 4ºC for 20 min (three prawns per group were sacrificed (3 × 6 = 18 prawns × 3 (triplicate) = 54 prawns). The supernatant was used as a crude enzyme source.

Total protease activity was determined by casein-hydrolysis method of Furne *et al.* (2005), one unit of enzyme activity represents the amount of enzyme required to liberate 1µg of tyrosine per minute under assay conditions. Amylase activity was determined by starch-hydrolysis method put forth by Bernfeld (1955), the specific activity of amylase was calculated as milligrams of maltose liberated per gram of protein per hour (mg/g/h). Lipase activity was determined by method of Furne *et al.* (2005), one unit of lipase activity was defined as the amount of free fatty acid released from triacylglycerol per unit time estimated by the amount of NaOH required to maintain pH constant and represented as mille equivalents of alkali consumed.

Estimations of concentrations of biochemical constituents

On the initial and final days, the concentrations of basic biochemical constituents, such as total protein, total carbohydrate and total lipid in the muscle of prawns were determined. Concentration of total protein was estimated by the method of Lowry *et al.* (1951) using ethanolic precipitated sample. Concentration of total carbohydrate was estimated by the method of Roe (1955) using TCA extracted sample.

Concentration of total lipid was extracted by following the method of Folch *et al*. (1957) and estimated by the method of Barnes and Blackstock (1973). For these parameters, tissues from five prawns were pooled together from each group to constitute a single observation and three such observations were made to fulfill the triplicate analysis (5 prawns/ group × 3 parameters = 15 prawns/ group × 3 replicates = 45 prawns).

Profiles of proteins, amino acids and fatty acids

These analyses were done in the best concentration of LBP incorporated (i.e. 4%) feed fed prawns. The muscle tissue samples were first defrosted in phosphate buffer (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4 and 2 mM KH_2PO_4, pH-7.4), homogenized under ice cooled condition and centrifuged at 1500 rpm at 4ºC for 5 min. The soluble protein content in supernatant was determined (Lowry *et al*., 1951). SDS-PAGE was performed (Laemmli, 1970) on vertical slab gel with 4% stacking and 10% separating gels. Protein markers consisting of six different molecular weights (Medox-Biotech Pvt. Ltd., India) from 116 – 14 kDa was also run simultaneously. The patterns were compared by using information on apparent molecular masses of bands and their intensity. The high performance thin layer chromatographic (HPTLC) method (Hess and Sherma, 2004) was adopted to analyze the profiles of amino acids. The gas chromatographic (GC) method (Nichols *et al*., 1993) was adopted to analyze the profiles of fatty acids.

Analysis of gut microbial colonization

The gut of control prawns and the gut of experimental prawns fed with the best concentration of LBP (i.e. 4%) were subjected to bacterial culture. The prawns were deactivated by keeping them in freezer at -20º C 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 homogenate were serially diluted up to 10^{-5} dilution individually. From this 0.5 ml of aliquots were taken and mixed with agar nutrient broth for 24 hr at 35ºC. 0.1 broth culture was seeded over the surface of freshly prepared nutrient agar plates and incubated at 37ºC for 24 hr. The different bacterial colonies were identified and they were confirmed through routine bacteriological tests (Holt *et al.,* 1996). 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. Yeast identification test was done by Bowman and Ahearn (1975). The bacterial/ yeast colonies were enumerated with the formula, Bacterial/ yeast count (CFU/g) = Number of colonies × Dilution factor/ Volume of sample (g).

Molecular characterization of gut microbes

On the final day of feeding experiment, the prawn gut was subjected to analyses of bacterial consortium. That is number of bacteria present in the gut of the prawn samples by using 16S rDNA based protocol (t-RFLP) (Chromous Biotech Pvt. Ltd., Bengaluru, India). This revealed that the probiotic product, LBP incorporated feed fed prawn showed 24 numbers of gut bacteria against 23 bacteria in the control. The most dominant bacterium in each sample was identified. The ~1.5kb rDNA fragment was amplified and cloned. One positive clone was sequenced bi-directionally and more than 80% sequence identity was done.

Agarose Gel Electrophoresis (AGE)

Genomic DNA was isolated from the gut of the prawns fed with LBP incorporated feed by using the Bacterial Genomic DNA Isolation Kit (RKT09). The isolated DNA was resolved and detected on AGE. 1X TAE was prepared and used as tank buffer (365 ml (i.e, 350, tank capacity +15 ml, boat capacity).150 mg agarose was dissolved in 15 ml TAE buffer (the agarose was melted in TAE buffer under micro oven for 1 minute). A drop of ethidium bromide was added and the gel was casted at room temperature. The boat was fixed into the tank filled with 350 ml of 1X TAE buffer. The sample DNA was mixed with loading dye containing Bromophenol blue and Glycerol in 2:6 ratio, and loaded carefully. The gel was supplied with 100 V DC for 30 min. The gel was safely removed and DNA profile on the gel was viewed under UV transilluminator.

PCR amplification condition

Amplification of DNA was done in Thermal Cycler ABI2720 by using primers, 27F5'-AGATTTGATCCTGGCTCAG-3' and 1492R5'-GGTTACCTTGTTACGACTT-3' of forward and reverse in natures respectively for 16S rDNA gene (Weisburg *et al*., 1991). PCR amplification was done on a 100-µl reaction mixture containing10X PCR buffer 10 µl, DNTP's 2 µl, M13F + M13R Primer 2 µl, Taq polymerase 0.50 µl, DNA 2 µl and MQ water 83.5 µl. The pre-running was 5 min at 94ºC. The denaturation was 35 cycles of 30 s each at 95ºC. The annealing was 30 s at 55ºC. The extension was 1 min 30 s at 72ºC, followed by 7 min at 72ºC for a final extension. The final product was stored in -20ºC for further usage. 2% Agarose was used to separate the PCR amplified DNA and visualized as described earlier.

DNA sequencing

DNA sequencing was done at Chromous Biotech, Bangalore, India by using ABI 3500 XL Genetic Analyzer. The sequencing reaction mix (10 µl) containing 4 µl Big Dye Terminator Ready Reaction Mix, 1 µl Template (100ng/µl), 2 µl Primer (10 pmol), 3 µl MilliQ Water, and the PCR conditions (25 cycles) are as follows, Initial denaturation at 96ºC for 1min, Denaturation at 96ºC for 10 sec, Hybridization at 50ºC for 5 sec and Elongation at 60ºC for 4 min.

Basic local alignment tool

BLAST is commonly used method for identification of DNA sequences (www.ncbi.nlm.nih.gov/BLAST). Based on the best sequence alignment to all or only to a part of the query sequence, a simple measurement of genetic distance was

calculated. It was used for species identification based on the similarity of the sequences.

Sequence alignment

Sequence alignment was performed by phylogenetic tree builder with System Software aligner. A distance matrix is generated using the Jukes-Cantor corrected distance model.

Neighbor joining tree

Weighbor is a weighted version of Neighbor Joining that gives significantly less weight to the longer distances in the distance matrix. The weights are based on variances and co-variances expected in a simple Jukes-Cantor model. It was used for constructing the phylogenetic tree by adopting MEGA v5.

Statistical analyses

One way analysis of variance (ANOVA) using SPSS (version 11.5) was applied to determine whether significant variations between control and treatments and between treatments existed. Difference between means were determined and compared by Duncan multiple range test (DMRT) and the significances are mentioned. The data are reported as means ± standard deviations.

RESULTS AND DISCUSSION

Survival and growth

The initial average body length and weight of prawn PL was 1.59±0.33 cm and 0.064±0.038 g respectively. After the feeding trial, the final length and final weight were found to be significantly ($P< 0.05$) increased in LBP incorporated diets fed *M. rosenbergii* when compared with control (Table 3). The survival rate (SR) and growth performance (LG, WG, SGR and PER) were significantly ($P<0.05$) increased in LBP incorporated diets fed prawns when compared with control (Table 4). Among different concentrations, 4% LBP incorporation was found to be the best including for FCR. The lower FCR recorded in experimental prawns reflects the superior quality of the diets formulated with incorporation of LBP. The better growth achieved in LBP incorporated diets fed prawns indicates that probiotics have the characteristic ability of growth promotion in *M. rosenbergii*.

Table 3. Morphometric data of *M. rosenbergii* PL fed with LBP incorporated diets

Parameters	Initial	Control	LBP incorporated diets fed prawns					F-Value
			1%	2%	3%	4%	5%	
Length (cm)	1.59 ±0.33	3.18^{f} ±0.47	3.58^{ef} ±0.30	4.12^{bcd}±0.34	4.26^{b} ±0.47	4.78^{a} ±0.25	3.94^{cde}±0.25	7.89
Weight (g)	0.064 ±0.03	0.39^{e} ±0.11	0.45^{de}±0.09	0.74^{bc} ±0.10	0.83^{bc} ±0.12	1.12^{a} ±0.36	0.51^{cd} ±0.19	9.25

Each value is mean ± standard deviation of three individual observations.
Mean values within the same row sharing different superscript are significantly different ($P<0.05$).
LBP, LactoBacil®$_{plus}$.

Table 4. Nutritional indices of *M. rosenbergii* PL fed with LBP incorporated diets

Parameter	Control	LBP incorporated diets fed prawns					F-value
		1%	2%	3%	4%	5%	
SR (%)	66.66^{h} ±2.51	73.33^{fg} ±2.00	80.00^{de} ±3.40	86.66^{bc} ±4.20	93.33^{a} ±3.60	76.66^{ef} ±2.40	22.13
LG (cm)	1.58^{f} ±0.1	1.99^{e} ±0.12	2.53^{cd} ±0.15	2.67^{c} ±0.11	3.19^{a} ±0.12	2.35^{d} ±0.14	35.01
WG (g)	0.32^{d} ±0.02	0.38^{cd} ±0.03	0.72^{bc} ±0.14	0.76^{b} ±0.20	1.05^{a} ±0.32	0.44^{cd} ±0.09	6.23
SGR (%)	0.86^{d} ±0.31	0.94^{cd} ±0.15	1.20^{abc} ±0.14	1.23^{abc} ±0.12	1.37^{a} ±0.10	0.99^{bcd} ±0.15	2.91
FCR (g)	5.47^{a} ±0.25	4.43^{b} ±0.31	1.70^{de} ±0.11	1.32^{de} ±0.15	0.81^{e} ±0.18	3.31^{c} ±0.21	48.81
PER (g)	0.20^{c} ±0.05	0.20^{c} ±0.04	0.28^{ab} ±0.03	0.29^{ab} ±0.01	0.31^{a} ±0.04	0.22b^{c} ±0.05	4.68

Each value is mean± standard deviation of three individual observations.
Mean values within the same row sharing different superscript are significantly different ($P<0.05$).
LBP, LactoBacil®$_{plus}$; SR, Survival Rate; LG, Length gain; WG, Weight gain, SGR, Specific growth rate; FCR, Feed conversion ratio; PER, Protein efficiency ratio.

Table 5. Activities of digestive enzymes (U/ mg protein) in *M. rosenbergii* PL fed with LBP incorporated diets

Parameter	Control	LBP incorporated diets fed prawns					F-value
		1%	2%	3%	4%	5%	
Protease	2.23^{c} ±0.015	2.51^{bc} ±0.2	2.76^{b} ±0.16	3.11^{a} ±0.18	3.27^{a} ±0.15	2.70^{b} ±0.21	6.75
Amylase	0.72^{d} ±0.02	1.83^{c} ±0.05	2.05^{b} ±0.06	2.39^{a} ±0.10	2.43^{a} ±0.09	1.99^{b} ±0.01	34.42
Lipase*	0.21^{e} ±0.017	0.42^{d} ±0.011	0.47^{c} ±0.013	0.51^{b} ±0.015	0.55^{a} ±0.012	0.45^{c} ±0.018	61.21

Each value is mean± standard deviation of three individual observations.
Mean values within the same column sharing the same superscript are not significantly different ($P > 0.05$)
LBP, LactoBacil®$_{plus}$; *, unit×10^{3}.

Similar results in SR, WG, SGR, FCR and PER have been reported in *M. rosenbergii* fed with *Bacillus* spp., KKU02, KKU03, Biogen®, Binifit™, *B. subtilis*, *L. sporogenes*, *Lactobacillus ceremoris*, *L. acidophillus* and yeast, *S. cerevisiae* incorporated diets (Suralikar and Sahu, 2001; Venkat *et al.,* 2004; Keysami *et al.,* 2007; Deeseenthum *et al.*, 2007; Shinde *et al.,* 2008; Saad *et al.*, 2009; Rinisha *et al.,* 2010; Seenivasan *et al*., 2011, 2012a-d, 2014a-c); in *M. amazonicum* juveniles fed with *S. cerevisiae* and yeast derivatives incorporated diets (Hisano *et al.,* 2008); *Fenneropenaus indicus* fed with *Bacillus* spp., incorporated diets (Ziaei-Nejad *et al.,* 2006); *L. vannamei* fed with *B. subtilis* UTM 126 and *S. boulardii* supplemented diets (Scholz *et al.,* 1999; Balcazar and Rojas-Luna, 2007); in *P. indicus* fed with commercial products Lacto-sacc™ and Protexin supplemented diets (Uma *et al.*, 1999; Javadi *et al.,* 2011); in *P. monodon* fed with *Bacillus* spp., supplemented diets (Boonthai *et al.,* 2011).

Growth is an index of water quality and nutrients provided. As far as freshwater prawn culture is concerned, there are many factors relate the growth and feeding activity, which include a functional digestive system to efficiently utilize the nutrients present in the food offered and also physiological conditions and the rearing environment (Lee and Lawrence, 1997; New and Valenti, 2000; Anderson and De-Silva, 2003). In aquaculture, SGR is a trait of great economic importance and the rapid growth speeds up the turnover of production, which is supported by good FCR and PER in which the native food protein is converted into body building protein.

Digestive enzymes

Activities of protease, amylase and lipase were found to be significantly increased ($P< 0.05$) in LBP incorporated diets fed prawns when compared with control. Among different concentrations, 4% LBP incorporation was found to be the best (Table 5). The increased digestive enzymes activity due to LBP may have improved digestion in experimental prawns. It is important to mention here that the metabolic byproducts of these probiotics might have acted as prebiotics, which can also be absorbed and helps in food conversion, which in turn ultimately produced better growth of *M. rosenbergii*. Therefore, the increased digestive enzymes activity directly or indirectly regulates growth in experimental prawns and regulating the body biochemical constituents. Similar improvement in activities of protease, amylase and lipase has been reported in *L. sporogenes*, *B. subtilis* and *S. cerevisiae* incorporated diet fed *M. rosenbergii* (Seenivasan *et al*., 2014c). The increase in activities of digestive enzymes has also been reported in *Bacillus* spp., incorporated diet fed Indian white shrimp, *F. indicus* (Ziaei-Nejad *et al.*, 2006) and common carp, *Cyprinus carpio* (Yanbo and Zirong, 2006; Wang and Xu, 2006). Suzer *et al.* (2008) reported increase in trypsin, amylase and lipase in *Lactobacillus* spp., incorporated diet fed sea bream, *Sparus aurata*. Wang (2011) reported significant elevation of amylase activity in the Grass carp, *Ctenopharyngodon idella* fed with *L. acidophilus* incorporated feeds.

Biochemical constituents

Concentrations of total protein, carbohydrate and lipid were found to be significantly increased ($P<0.05$) in LBP incorporated diets fed prawn groups when compared with control. Among different concentrations, 4% LBP incorporation was found to be the best (Table 6). Since the level of total protein, carbohydrate and lipid was elevated, the basic physiology was in good state in experimental prawns. This suggests that the probiotics present in LBP have the characteristics ability to promote the maintenance of basic biochemical constituents and leads to better growth of *M. rosenbergii*.

Similar elevation in basic biochemical constituents have been reported in *M. rosenbergii* fed with *L. sporogenes* and *L. acidophilus*; Biogen®; Binifit™; *L. sporogenes* bio-encapsulated *Artemia*; *L. sporogenes, B. subtilis* and yeast*, S. cerevisiae*; incorporated feeds (Venkat *et al.*, 2004; Saad *et al.,* 2009; Seenivasan *et al.,* 2011, 2012a-d, 2014a-c). Increase in biochemical composition has also been reported in marine prawn, *L. vannamei* fed with *Bacillus* spp., supplemented diet (Yu *et al.,* 2009). Fernandez *et al.* (2011) reported that Lactic acid bacteria enhanced the crude protein and ash content in juveniles of *P. indicus*.

Body biochemical composition is a good indicator of the physiological status of an organism. One of the major requirements of prawn culture is the transformation of dietary protein into tissue protein. Carbohydrates are the first among the organic nutrients to be utilized to generate required energy. They serve as precursors for the dispensable amino acids and some nutrients, which are metabolic intermediates necessary for growth (NRC, 1993). They exist both in free and in bound state along with proteins as protein–bound sugars and glycogen. Lipids are the best source of energy producers of the body through metabolism. They provide a source of indispensable nutrients and act as carriers of certain non-fat nutrients, like the fat soluble vitamins, such as A, D, E and K (New, 1986; Ricardo *et al*., 2003).

Profiles of proteins, amino acids and fatty acids

Polypeptide bands of molecular weight between 116-14 kDa were resolved in the muscle tissue of prawns (Fig 1). Generally, there were ten Coomassie blue stained protein bands (116, 51, 45, 36, 29, 27, 20, 18, 16 and 14 kDa) calculated against the standard markers of 116, 66, 45, 29, 20 and 14 kDa, which represent β-galactosidase, Bovine serum albumin, ovalbumin, carbonic anhydrase, soyabean trypsin inhibitor and lysozyme respectively. In LBP incorporated feed fed prawn sample, the staining intensity of 36, 29 and 18 kDa regions was found to be higher when compared with control feed fed prawns. It indicates that the LBP have influence on protein quality of the prawn. It is corroborate with the increased total protein content of LBP incorporated feed fed prawns (Table 6).

Thirteen amino acids (essential category: valine, lysine, threonine, isoleucine, tyrosine, arginine, histidine and leucine; non-essential category: glutamic acid, serine, proline, glysine and alanine) were deducted in *M. rosenbergii* (Table 7). In control diet fed prawns, valine, thyrosin and histidine were in traces. In the case of 4% LBP incorporated diet fed prawns valine, lysine, thyrosin and histidine were in traces. Generally the content of all the essential and non-essential amino acids were found to be significantly higher ($P<0.05$) in LBP

incorporated diet fed prawns when compared with control. However, lysine was highly present in control diet fed prawns. Increase in amino acid profile has been reported in *M. rosenbergii* fed with *L. sporogenes, B. subtilis, S. cerevisiae* supplemented diets (Seenivasan *et al*., 2014c). In the present study, the LBP incorporated diet fed prawns attained better amino acid profile and thus better protein synthesis and muscle growth was achieved in *M. rosenbergii*.

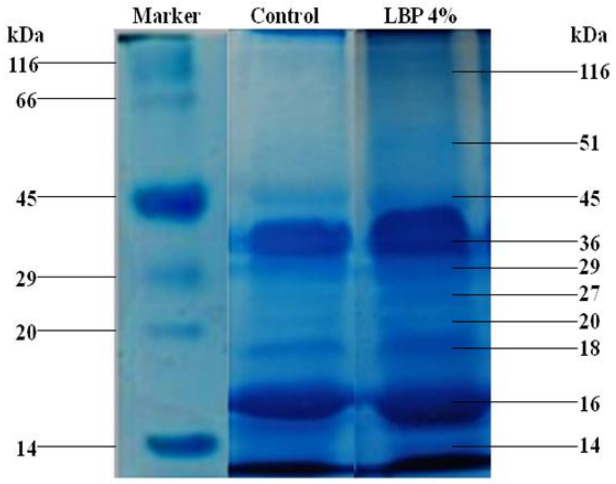

Fig. 1. SDS-PAGE pattern of muscle protein of *M. rosenbergii* PL fed with control and LBP incorporated diets

Marker: 116 kDa, β-galactosidase; 66 kDa, bovine serum albumin; 45 kDa, ovalbumin; 29 kDa, carbonic anhydrase; 20 kDa, soyabean trypsin inhibitor; 14 kDa, lysozyme.
LBP, LactoBacil®plus.

Twelve fatty acids (saturated category: lauric acid, myristic acid, palmitic acid, stearic acid, behenic acid, lignoceric and elaidic acid; un-saturated category: oleic acid, linoleic acid, linolenic acid, EPA and DHA) were deducted in *M. rosenbergii* (Table 8). The linoleic (n-6, polyunsaturated, PUFA) and EPA (highly unsaturated, HUFA) were significantly higher in 4% LBP incorporated diet fed prawns when compared with control diet fed prawns ($P<0.05$). Whereas, the oleic (monounsaturated) and linolenic (n-3, PUFA) were not significantly lower in 4% LBP incorporated diet fed prawns when compared with control diet fed prawns. However, DHA (HUFA) was highly present in control diet fed prawns, which was in traces in LBP incorporated diet fed prawns. In the saturated category, palmitic acid was highly present in the control prawns, whereas, behenic and lignoceric acids were highly present in experimental prawns ($P<0.05$). Other saturated fatty acids, myristic and stearic acids were also significantly higher in experimental prawns ($P<0.05$).

In this study, majority of the amino acids were quantitatively higher in LBP incorporated diet fed prawns then in control prawns. The presence of higher amount of saturated fatty acids (myristic acid, stearic acid, behenic acid and lignoceric acid) as well as the unsaturated fatty acids (linoleic acid and EPA) in LBP incorporated diets fed prawn. Therefore, it is clear that the probiotic nutrients of LBP have significantly regulates protein, amino acid and fatty acid profiles, which led to better survival, growth and carcass biochemical constituents in *M. rosenbergii*.

Table 6. Concentrations of biochemical constituents (mg/g wet wt.) in *M. rosenbergii* PL fed with LBP incorporated diets

Parameters	Control	LBP incorporated diets fed prawns					F-Value
		1%	2%	3%	4%	5%	
Total Protein	197.64^{i} ±7.56	222.47^{h} ±10.39	274.53^{c} ±11.38	299.59^{ab} ±10.43	315.26^{a} ±9.34	234.83^{fg} ±9.45	36.68
Total Carbohydrate	42.95^{c} ±3.71	46.47^{bc} ±4.71	50.70^{abc} ±4.51	54.22^{ab} ±5.21	58.09^{a} ±3.92	50.00^{bc} ±3.21	3.79
Total Lipid	19.48^{h} ±2.69	21.53^{fgh} ±1.32	28.71^{bcde} ±2.00	31.79^{abc} ±2.34	34.87^{a} ±3.1	26.66^{def} ±2.91	12.13

Each value is mean± standard deviation of three individual observations. Mean values within the same row sharing different superscript are significantly different ($P<0.05$). LBP, LactoBacil®$_{plus}$.

Table 7. Profiles of amino acids in *M. rosenbergii* PL fed with 4% LBP incorporated diets

Amino acid (g/ 100 g dry wt.)		Control	4% LBP	F- Value
Essential	Valine	trace	trace	-
	Lysine	1.75 ± 0.26	trace	-
	Threonine	0.63 ± 0.022^{b}	0.80 ± 0.015^{a}	35.46
	Isoleucine	0.67 ± 0.02^{c}	1.05 ± 0.016^{a}	200
	Tyrosine	trace	trace	-
	Arginine	2.00 ± 0.40^{b}	5.79 ± 0.42^{a}	119.36
	Histidine	trace	trace	-
	Leucine	2.37 ± 0.20^{c}	4.34 ± 0.18^{a}	100.70
Non-essential	Glutamine	1.01 ± 0.044^{b}	1.08 ± 0.017^{a}	15.45
	Serine	0.51 ± 0.04^{b}	0.76 ± 0.09^{a}	11.53
	Proline	0.72 ± 0.013^{c}	0.83 ±0.026^{a}	113.49
	Glycine	0.41 ± 0.017^{c}	0.55± 0.014^{a}	38.24
	Alanine	0.44 ±0.02^{c}	0.58 ± 0.016^{a}	52.11

Each value is mean± standard deviation of three individual observations. Mean values within the same row sharing different superscript are significantly different ($P<0.05$). LBP, LactoBacil®$_{plus}$.

Gut microbial population

Analysis of gut microflora of the control prawn showed presence of *Bacillus* spp., *Pseudomonas* spp., *E. coli* and *Streptococcus* spp., (Tables 9 and 10). In the gut of experimental prawns fed with 4% LBP incorporated diets, the presence of *Pseudomonas* spp., was replaced by establishment of colonies of *L. acidophilus, L. rhamnosus, B. longum, B. bifidum* and *S. boulardii* (40×10^{-4}, 42.52×10^{-4}, 40.24×10^{-4}, 37.1×10^{-4} and 11.1×10^{-4} CFU cells respectively; total: 170.96×10^{-4} CFU cells). In addition to this establishment of *E. coli* (22×10^{-4} CFU cells) was also found to be present in the experimental prawn gut. These strains were absent in the case of control prawns. The presence of colonies of these bacterial isolates were identified and confirmed through routine biochemical tests (Table 11). In the present study gut microbial analysis of experimental prawns showed the presence of probiotic bacteria which were originally present in the probiotic product, LBP. This suggests that the selected probiotic strain can survive and withstand the conditions of the prawn digestive tract. Instead, the presence of these bacterial colonies in the gut of *M. rosenbergii* may aid for better digestion, survival and growth.

Adhesion and colonization of probiotic bacteria in the gastro intestinal tract of the host is believed to be one of the essential features required for providing their health benefits (Bernet *et al*., 1994). An important aspect of probiotic bacterial function is the protection of the host gastrointestinal microenvironment from invading pathogen adhesion to tissue surface by competitive exclusion during the initial stages of pathogenic infection (Reid *et al*., 1990; Krovacek *et al*., 1987; Montes and Pugh, 1993). In this study such a mechanism may be activated by the probiotics of LBP to exclude *Pseudomonas* spp.

Bacterial species present in the aquatic environment influence the composition of the gut microbiota and vice versa. Cai *et al.* (1999) reported the presence of three bacterial species, *Lactococcus garvieae*, *Pediococcus acidilactici* and *Enterococcus faecium* in the gut of *M. rosenbergii*. The bacterial species present in the intestinal tract generally seem to be those from the environment or the diet (Cahill, 1990). Diet influenced establishment of gut microbial colony have been reports in *M. rosenbergii* for *L. sporogenes, B. subtilis, S. cerevisiae* and *L. acidophilus* (Venkat *et al.,* 2004; Seenivasan *et al.,* 2012a-d, 2013, 2014a-b); in *P. monodon* for *Bacillus* S11 (Rengpipat *et al.,* 2000).

Table 8. Profiles of fatty acids in *M. rosenbergii* PL fed with 4% LBP incorporated diets

Fatty acids (%)		Control	4% LBP	F-value
Saturated	Lauric acid	4.53±0.12[a]	4.45±0.21[a]	647.78
	Myristic acid	4.05±0.10[c]	7.13±0.22[b]	675.718
	Palmitic acid	54.49±3.24[a]	4.45±0.11[b]	726.291
	Stearic acid	3.58±0.22[b]	5.03±0.26[a]	51.060
	Behenic acid	5.85±0.64[c]	46.51±3.24[a]	232.14
	Lignoceric	5.23±0.18[c]	26.09±2.04[a]	179.533
	Elaidic acid	4.75±0.16[a]	4.33±0.22[c]	4.544
Unsaturated	Oleic acid	6.09±0.34[b]	5.28±0.17[b]	47.449
	Linoleic acid	0.33±0.10[c]	4.16±0.11[b]	1.392
	Linolenic acid	4.75±0.13[a]	4.33±0.33[a]	1.810
	EPA	1.33±0.12[b]	5.35±0.22[a]	771.99
	DHA	3.57±0.11	trace	--

Each value is mean± standard deviation of three individual observations. Mean values within the same row sharing different superscript are significantly different ($P<0.05$). LBP, LactoBacil®plus; EPA, ecosapentanoic acid; DHA, decosahexanoic acid.

Table 9. Percentage composition and total viable count of bacterial flora in the gut of control and 4% LBP incorporated diets fed *M. rosenbergii* PL

Samples	Dilution	Isolate Name	Composition (%)	Total percentage
Control	10^{-4}	*Bacillus cereus*	30.13	
		Pseudomonas spp.,	20.55	
		E. coli	21.23	97.36%
		Streptococcus faecium	25.45	
4% LBP	10^{-4}	*L. acidophilus*	20.00	
		L. rhamnosus	21.26	
		E. coli	11.00	96.48%
		B. longum	20.12	
		B. bifidum	18.55	
		S. boulardii	5.55	

LBP, LactoBacil®plus.

Table 10. Overall result of microbial load in the gut of control and 4% LBP incorporated diets fed *M. rosenbergii* PL

Isolated species	Control	4% LBP
Bacillus sp.	P (60.26×10^{-4} CFU cells)	P
Pseudomonas sp.	P (41.10×10^{-4} CFU cells)	A
E. coli	P (42.46×10^{-4} CFU cells)	P
Streptococcus sp.	P (50.90×10^{-4} CFU cells)	P
L. acidophilus	A	P (40×10^{-4} CFU cells)
L. rhamnosus	A	P (42.52×10^{-4} CFU cells)
B. longum	A	P (40.24×10^{-4} CFU cells)
B. bifidum	A	P (37.1×10^{-4} CFU cells)
S. boulardii	A	P (11.1×10^{-4} CFU cells)

LBP, LactoBacil®plus; A, Absent; P, Present

Table 11. Confirmative results of different microflora by morphological, biochemical and physiological tests in control and 4% LBP incorporated diets fed *M. rosenbergii* PL gut

Tests	Control prawn gut				Experimental prawn gut					
	B.C	*P.S.*	*E.C*	*S.F*	*L.C*	*E.C*	*L.R*	*B.L*	*B.B*	*S.B*
Gram's staining	+	-	-	+	+	-	+	+	+	-Nil-
Motility test	+	+	+	+	-	+	-	-	-	-Nil-
Indole test	-	-	+	-	-	+	-	-	-	-Nil-
Methyl red test	-	-	+	-	+	+	-	+	+	-Nil-
VP test	-	+	-	+	-	-	-	+	-	-Nil-
Citrate utilization test	+	+	-	+	-	-	+	+	-	-Nil-
Starch hydrolases	+	-	+	+	+	+	+	+	+	-Nil-
Gelatin hydrolases	+	+	+	+	+	+	-	-	+	-Nil-
Nitrate reduction test	+	-	+	+	-	+	-	-	-	-Nil-
Oxidase test	-	+	+	-	-	+	-	-	-	-Nil-
Catalase test	+	+	-	-	+	-	-	-	-	-Nil-
Glucose test	A	A	A	A	A	A	A	A	A	+
Lactose test	A	NA	A	A	A	A	A	A	A	-
Sucrose test	A	A	A	A	A	A	A	A	A	+
Manitol test	A	A	A	A	NA	A	A	A	A	-
Maltose test	A	NA	A	A	A	A	A	A	NA	+

B.C, *B. cereus*; P.S, *Pseudomonas* sp.; E.C, *E. coli*; S.F, *S. faecium*; L.C, *L. acidophilus*; L.R, *L. rhamnosus*; B.L, *B. longum*; B.B, *B. bifidum*; S.B, *S. boulardii*.+, Positive; -, Negative; A, Acid production; NA, No acid production.

Molecular characterization of gut microbes

Genomic DNA and 16S rDNA gene

The isolated genomic DNA was resolved in 1% AGE, and they have >10kb length (Fig 2). The amplification of 16S rDNA gene using forward (27 F) and reverse (1492 R) primers, and the resulted ~1450bp mixed products have been resolved in 2% AGE (Fig 3). These products showed 1490 bp sequences in the control prawn gut and 1355 bp sequences in the experimental prawn gut (Figs 4 and 5).

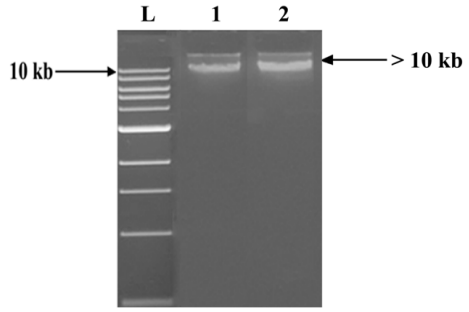

Fig. 2. 1% AGE indicates approximately 10kb genomic DNA from the gut of *M. rosenbergii* PL fed with control and 4% LBP incorporated diets. L, 10kb ladder; 1, control; 2, LBP (LactoBacil®plus)

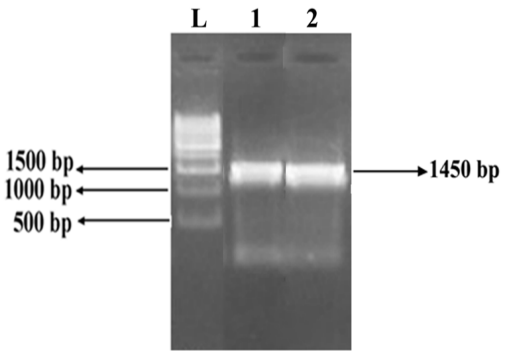

Fig. 3. 2% AGE indicates 1450 base pair PCR amplified product of bacterial genome from the gut of *M. rosenbergii* PL fed with control and 4% LBP diets L, 100 bp ladder; 1, control; 2, LBP (LactoBacil®plus).

TACGGTTACCTTGTTACGACTTAACCCCAGTCATGAATC
CCACCGTGGTAAGCGCCCTCCTTGCGGTTAGGCTACCTA
CTTCTGGTGAAACCCACTCCCATGGTTTGACGGGCGGTG
TGTACAAGACCCGGGAACGTATTCACCGTGACATTCTGA
TCCACGATTACTAGCGATTCCGACTTCATGGAGTCGAGT
TGCAGACTCCAATCCGGACTACGATCGGTTTTATGGGAT
TGGCTCCACCTCGCGGCTTGGCAACCCTTTGTACCGACC
ATTGTATGACGTGTGAAGCCCTACCCATAAGGGCCATGA
GGACTTGGCGTCATCCCCACCTTCCTCCGGTTTGTCACCG
GCAGTCTCGCTAAAGTGCCCAACTTAATGATGGCAATTA
ACGACAAGGGTTGCGCTCGTTGCGGGACTTAACCCAACA
TCTCACGACACGAGCTGACGACAGCCATGCAGCACCTGT
GTCCACTTTCTCTTTCGAGCACCTAATGCATCTCTGCTTC
GTTAGTGGCATGTCAAGGGTAGGTAAGGTTTTTCGCGTT
GCATCGAATTAATCCACATCATCCACCGCTTGTGCGGGT
CCCCGTCAATTCATTTGAGTTTTAATCTTGCGACCGTACT
CCCCAGGCGGTCAACTTCACGCGTTAGCTTCGTTACTAA
GCCAATGAAGGCCCAACAACCAGTTGACATCGTTTAGGG
CGTGGACTACCAGGGTATCTAATCCTGTTTGCTCCCCAC
GCTTTCGTACATGAGCGTCAGTGTATCCAAGGAGCCGCC
TTTCGCCACCCGGTATCCCTCCACATCTCTACGCACTTCA
CTGCTACACGTGGAATTCTACCTCCCTCTGACACACTCTA
GTCTGACAGTTACAATCGCAGTTCCCCAAGTTAAGCTCG
GGGATTTCACGACTGTCTTATCAGACCGCCTGCGCACGC
TTTACGCCCAGTAATTCCGATTAACGCTCGCACCCTACG
TATTACCGCGGCTGCTGGCACGTAGTTAGCCGGTGCTTA
TTCTTCAGGTACCGTCATCCCTCAGTGATATTAGCACTAA
GGATTTCTTCCCTGACAAAAGAGCTTTACAACCCGAAGG
CCTTCTTCACTCACGCGGCATTGCTGGATCAGGGTTGCC
CCCATTGTCCAAAATTCCCCACTGCTGCCTCCCGTAGGA
GTCTGGACCGTGTCTCAGTTCCAGTGTGGCGGATCGTCC
TCTCAAACCAGCTAGGGATCGTCGCCTTGGTGAGCCGTT
ACCTCACCAACTAGCTAATCCCACGTAGGCGCATCCGAT
AGCATGTGGCCCGAAGGTCCCACACTTTGGTCCGTAGAC
ATTATGTGGTATTAACAGTCGTTTCCAACTGGTATCCCCC
TCTGTCGGGTAGCCTCCTACGCATTACTCACCCGTCCGCC
GCTAGGTCCGAAAACCCCGCTCGACTTGCATGTGTTAGG
CCTGCCGCCAGCGTTCAATCTGAGCCAGGATCAAACTCT

Fig. 4. BLAST identification of a 1490 bp sequence of microbes from the gut of control diet fed *M. rosenbergii* PL

AGAGTTTGATCATGGCTCAGATTGAACGCTGGCGGCAGGCC
TAACACATGCAAGTCGAGCGGGGTTTTCGGACCTAGCGGCG
GACGGGTGAGTAATGCGTAGGAAGCTACCCGACAGAGGGG
GATACCAGTTGGAAACGACTGTTAATACCACATAATGTCTA
CGGACCAAAGTGTGGGACCTTCGGGCCACATGCTATCGGAT
GCGCCTACGTGGGATTAGCTAGTTGGTGAGGTAACGGCTCA
CCAAGGCGACGATCCCTAGCTGGTTTGAGAGGACGATCCGC
CACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGG
CAGCAGTGGGGAATTTTGGACAATGGGGGCAACCCTGATCC
AGCAATGCCGCGTGAGTGAAGAAGGCCTTCGGGTTGTAAAG
CTCTTTTGTCAGGGAAGAAATCCTTAGTGCTAATATCACTGA
GGGATGACGGTACCTGAAGAATAAGCACCGGCTAACTACGT
GCCAGCAGCCGCGGTAATACGTAGGGTGCGAGCGTTAATCG
GAATTACTGGGCGTAAAGCGTGCGCAGGCGGTCTGATAAGA
CAGTCGTGAAATCCCCGAGCTTAACTTGGGAACTGCGATTG
TAACTGTCAGACTAGAGTGTGTCAGAGGGAGGTAGAATTCC
ACGTGTAGCAGTGAAGTGCGTAGAGATGTGGAGGAATACC
GGTGGCGAAAGCGGCCTCCTGGGATAACACTGACGCTCATG
TACGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGT
AGTCCACGCCCTAAACGATGTCAACTGGTTGTTGGGCCTTC
ATTGGCTTAGTAACGAAGCTAACGCGTGAAGTTGACCGCCT
GGGGAGTACGGTCGCAAGATTAAAACTCAAATGAATTGAC
GGGGACCCGCACAAGCGGTGGATGATGTGGATTAATTCGAT
GCAACGCGAAAAACCTTACCTACCCTTGACATGCCACTAAC
GAAGCAGAGATGCATTAGGTGCTCGAAAGAGAAAGTGGAC
ACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATG
TTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTCGTTAAT
TGCCATCATTAAGTTGGGCACTTTAGCGAGGCGGCGGGGTG
ACAAACCGGAGGAAGGTGGGGATGACGCCAAGTCCTCATG
GCCCTTATGGGTAGGGCTTCACACGTCATACAATGGTCGGT
ACAAAGGGTTGCCAAGCCGCGAGGTGGAGCCAATCCCATA
AAACCGATCGTAGTCCGGATTGGAGTCTGCAACTCGACTCC
ATGAAGTCGGAATCGCTAGTAATCGTGGATCAGAATGTCAC
GGTGAATAC

Fig. 5. BLAST identification of a 1355 bp sequence of microbes from the gut of 4 % LBP incorporated diet fed *M. rosenbergii* PL

Similarity search

The BLAST similarity search with NCBI database revealed that the bacterial consortium of control prawn gut showed 98% similarity with *Ralstonia* (genes of proteobacterium), and the LBP incorporated feed fed prawn gut bacterial consortium showed 93% similarity with *Ralstonia* (genes of proteobacterium). The alignment scores and GenBank accession numbers are presented in Tables 12-14.

Phylogenetic analysis

The phylogenetic tree topology for dominant bacterial species retrieved against the sequence of control prawn gut bacterial consortium and LBP incorporated prawn gut bacterial consortium are presented in Figs 6 and 7. The sequence alignment and analysis of control prawns revealed that the sequence of bacterial consortium closely matched with 10 microbial species available in the GenBank (Fig 6). Among these the very closest bacterium was found to be uncultured beta Proteobacterium; ATB-LH-6158 (GenBank entry: FJ535157). The next closest homologue was found to be uncultured Burkholderiales bacterium; 4.13m3 (GenBank entry: JN695853). Similarly, the LBP incorporated feed fed prawns gut revealed that the sequence of bacterial consortium also closely matched with10 microbial species available in the GenBank (Fig 7). Among these the very closest bacterium was found to be uncultured beta Proteobacterium; ATB-LH-6158 (GenBank entry: FJ535157), and the next closest homologue was found to be uncultured beta Proteobacterium; 3.29m38 (GenBank entry: JN679109). In this study, the results revealed that the identified gut microbes were differed from originally incorporated probiotics and unidentified bacterial species were present.

Table 12. BLAST identification of dominant bacterial genome (16S rDNA) present in the gut of *M. rosenbergii* PL fed with control and 4% LBP incorporated diets

Prawn group	Identified bacteria	Similarity score	Expected	Identities	Gaps	Strand
Control	*Ralstonia* (genes of proteobacterium)	2180 bits (1180)	0.0	1245/1276(98%)	5/1276 (0%)	Plus/Minus
4% LBP	*Ralstonia* (genes of proteobacterium)	1949 bits (1055)	0.0	1276/1379 (93%)	30/1379 (2%)	Plus/Plus

LBP, LactoBacil®plus.

Table 13. Alignment view and distance matrix of dominant bacterial genome (16S rDNA) present in the gut of *M. rosenbergii* PL fed with control diet

Name of the organism	NCBI accession number	Similarity score
Uncultured beta proteobacterium: AT-LH-6158	FJ535157	0.949
Uncultured beta proteobacterium: 3.29m38	JN679109	0.945
Uncultured Burkholderiales bacterium: 4:13m3	JN695853	0.945
Uncultured Burkholderiales bacterium: 4.6m1	JN679136	0.943
Uncultured bacterium: HTG2	AF418961	0.935
Uncultured Burkholderiales bacterium: LC01	JF733411	0.932
Unidentified bacterium: T34	Z93984	0.928
Uncultured bacterium: ncd335f10c1	HM318293	0.927
Uncultured bacterium: SL-119	JF497786	0.927
Uncultured *Ralstonia* sp.: AV-4S-M03	EU341170	0.927

Table 14. Alignment view and distance matrix of dominant bacterial genome (16S rDNA) present in the gut of *M. rosenbergii* PL fed with 4% LBP incorporated diet

Name of the organism	NCBI accession number	Similarity score
Uncultured beta proteobacterium: AT-LH-6158	FJ535157	0.951
Uncultured beta proteobacterium: 3.29m38	JN679109	0.946
Uncultured Burkholderiales bacterium: 4:13m3	JN695853	0.945
Uncultured Burkholderiales bacterium: 4.6m1	JN679136	0.943
Uncultured bacterium: HTG2	AF418961	0.935
Uncultured Burkholderiales bacterium: LC01	JF733411	0.931
Unidentified bacterium: T34	Z93984	0.930
Uncultured bacterium: ncd335f10c1	HM318293	0.928
Uncultured bacterium: SL-119	JF497786	0.928
Uncultured *Ralstonia* sp.: AV-4S-M03	EU341170	0.927

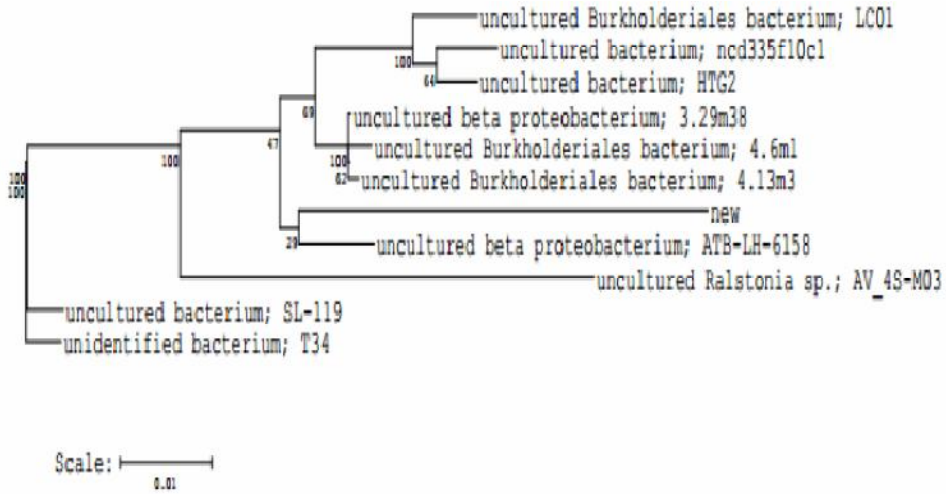

Fig. 6. Phylogenetic tree of identified bacteria form the gut of control diet fed *M. rosenbergii* PL. Neighbor-joining tree (non linear) of 16S rDNA sequence divergence (Jukes-Cantor) of bacteria. Numbers next to internal branches are Bootstrap values

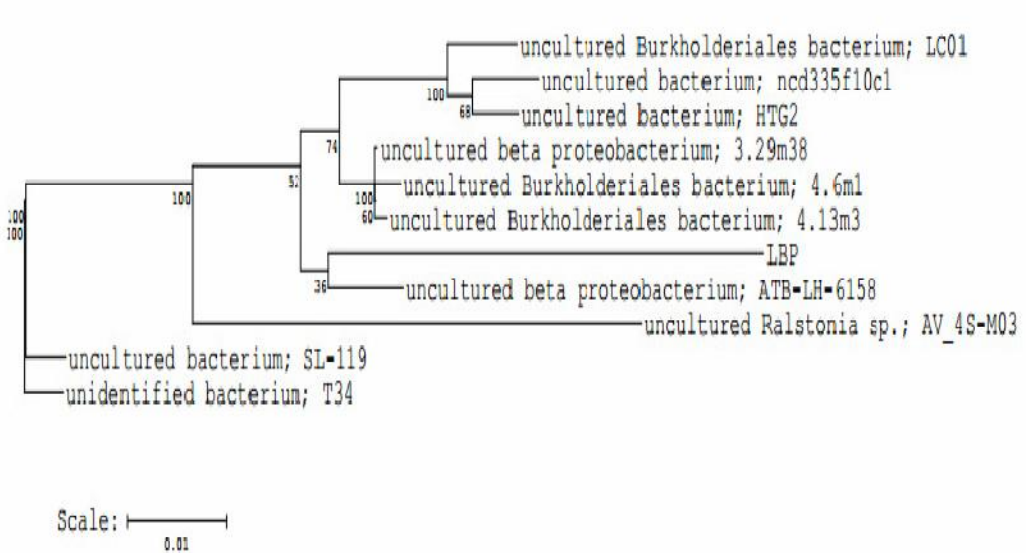

Fig. 7. Phylogenetic tree of identified bacteria from the gut of 4% LBP incorporated diet fed *M. rosenbergii* PL. Neighbor-joining tree (non linear) of 16S rDNA sequence divergence (Jukes-Cantor) of bacteria. Numbers next to internal branches are Bootstrap

Therefore, this part needs further clarification and requires repetition of cloning procedure.

In conclusion, the selected probiotic product, LBP at optimized concentration of 4% was found to be enhanced the survival, growth, nutritional indices, digestives enzymes activity, tissue biochemical constituents and gut microbial diversity of *M. rosenbergii*. Beyond the optimized concentration, it showed decreases in the above said parameters studied due to the over dosage. Therefore, the probiotic product, LBP can be recommended to incorporate in aqua feed formulations. However, study with the optimized concentration of LBP needs to be evaluated under field condition with the candidate species *M. rosenbergii*.

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