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Influence of combined probiotics *Lactobacillus sporogenes* and *Bacillus subtilis* on survival, growth, biochemical changes and energy utilization performance of *Macrobrachium rosenbergii* (De Man 1879) post larvae

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Abstract
A 90-day feeding experiment was studied to determine the influence of the bacterial combination *Lactobacillus sporogenes* and *Bacillus subtilis* (LS+BS) on survival, growth, biochemical constituents and energy utilization performance of the freshwater prawn *Macrobrachium rosenbergii* post larvae (PL). Experimental diets were the same, except for the variation in probiotic levels. The probiotics LS+BS (4:3) were used at 0%, 1%, 2%, 3% and 4% inclusion rates in the experimental diets. After the feeding trail, the growth parameters of the PLs, such as survival, weight gain, specific growth rate, feed conversion efficiency and protein efficiency rate were significantly ($P<0.05$) higher in 4% LS+BS incorporated diet as compared with control. The food conversion ratio was significantly ($P<0.05$) lower in 4% LS+BS incorporated diet. The biochemical composition of the total protein, amino acid, carbohydrate and lipid ash content were significantly ($P<0.05$) higher in 4% LS+BS incorporated diet. However, insignificant difference was recorded in moisture content between control and experimental groups. The energy utilization parameters, such as feeding rate, absorption rate, conversion rate and metabolic rate were significantly ($P<0.05$) higher in 4% LS+BS incorporated diet fed PL. These results revealed the benefits of the incorporation of the probiotic (LS+BS) in aqua feed for *M. rosenbergii* PL.

Keywords: Biochemical composition, *B. subtilis,* energy utilization, growth, *L. sporogenes*

INTRODUCTION

The culture of freshwater prawn offers tremendous scope to meet the awe some challenge of providing adequate levels of nutritious food to the growing human population [1]. The giant freshwater prawn (*Macrobrachium rosenbergii*) is a species, which plays an important role in the aquaculture and fisheries industry. Parker [2] coined the term probiotic and defined the term as "organisms and substances which contribute to intestinal microbial balance". Probiotics can also be considered as microbes to improve the nutritive value of an animal feed [31]. A number of studies have shown that a single probiotic ingredient can improve the growth performance of the freshwater prawns and shrimps [4-14]. The present investigation was conducted to determine the effects of combined probiotics, *L. sporogenes* and *B. subtilis* (4:3) on survival, growth, biochemical constituents and energy utilization of the freshwater prawn *M. rosenbergii* post larvae (PL).

MATERIALS AND METHODS

The post larvae of freshwater prawn, *M. rosenbergii* (PL 15) were purchased from a Happy Bay Annexe, Kanchipuram, Tamilnadu, India and were stocked in a cement tank (1000 L) filled with freshwater. The PL were acclimatised at ambient laboratory conditions for 15 days (up to PL 30) and starved for 24 h before the commencement of the feeding experiment. The experimental water had these physicochemical parameters: pH 7.00±0.30; total dissolved solids 0.90±0.08 g/L^{-1}; dissolved oxygen 7.10±0.10 mg/L^{-1}; BOD 4.10±2.60 mg/L^{-1}; COD 12.00±10.00 mg/L^{-1} and ammonia 0.098±0.018 mg/L^{-1}.

Diet preparation

The composition of the experimental diets is given in Table 1. The probiotics, *L. sporogenes* (Uni-Sankyo Ltd., Maharashtra, India) and *B. subtilis* (Tablets, India Ltd), one gram of lyophilized powders contains $15x10^7$ and $10x10^7$cfu cells respectively. The probiotics, LS+BS (4:3) were incorporated in to the test diets at five different concentrations individually 0% (control), 1%, 2%, 3% and 4% respectively. Diet formulation was done basically by "Pearson's square-method" using determined values of 40% protein content (Table 1). The proportion of each ingredient was calculated precisely providing allowance for the premix. The dough was steam cooked and cooled to room temperature. After that different concentration of LS+BS (4:3) was mixed with the dough and the diets were pelletized separately with a locally made (Kolkata, India) hand pelletizer. The pellets were dried in a thermostatic oven (M/s Modern Industrial, Mumbai, India) at 40^0 C until it reached constant weight and stored in airtight jars at room temperature.

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Table 1. Ingredients and proximate composition of prepared diets

Ingredients (%)	Control diet	Experimental diets (*L. sporogenes*+*B. subtilis* incorporated)			
		1%	2%	3%	4%
Fish meal	33.84	33.84	33.84	34.84	35.84
Ground nut oil cake	25.00	25.00	25.00	25.00	24.00
Soybean meal	24.00	24.00	23.00	21.00	20.00
Corn flour	4.00	3.00	3.00	3.00	3.00
Egg albumin	5.06	5.06	5.06	5.06	5.06
Tapioca flour	5.10	5.10	5.10	5.10	5.10
Cod liver oil	2.00	2.00	2.00	2.00	2.00
Vitamin B-complex mix	1.00	1.00	1.00	1.00	1.00
Probiotics (LS+BS)	0.00	1.00	2.00	3.00	4.00
Proximate composition					
Protein (%)	40.10	40.00	39.63	39.52	39.40
Carbohydrate (%)	21.76	21.10	20.71	20.01	19.50
Lipid (%)	9.28	9.24	9.17	9.08	8.90
Ash (%)	14.00	13.00	12.00	13.00	14.00
Moisture (%)	9.50	9.90	9.40	9.10	9.10
Digestible energy (k.cal/kg)	3296.86	3262.52	3228.17	3193.83	3159.49

Feeding experiment

M. rosenbergii (PL-30) with the length and weight range of 1.61±0.05 cm and 0.25±0.04 g respectively were used for feeding experiment. 40 PL for each diet in triplicate were maintained in plastic tanks with 20 L water. The PLs were maintained at the stocking density of 2/l. One group served as control, with 0% probiotics. The experimental groups were fed twice a day (6:00 am and 6:00 pm) with the respective concentration of LS+BS (4:3) incorporated diets. . The daily ration was given at the rate of 10% of the body weight of PL with two equal half throughout the experimental period. The unfed feed, faeces and moult if any, were collected after the respective hours of feeding. The feeding experiment was prolonged for 90 days; mild aeration was given continuously in order to maintain the optimal oxygen level.

Growth study

After the feeding trial, the survival rate (SR), weight gain (WG), specific growth rate (SGR), feed conversion rate (FCR), feed conversion efficiency (FCE) and protein efficiency rate (PER) were individually determined by the following equations [15].

Survival (%) = Total No. of live animals/Total No. of initial animals x 100
Weight gain (g) = Final weight (g) – Initial weight (g)
SGR (%) = log w_2 – log w_1/ t x100 (where, w_1 & w_2 = Initial and Final weight respectively (g), and t = Total number of experimental days)
Feed conversion rate (g) = Total Feed intake (g)/ Total weight gain of the prawn (g)
Feed conversion efficiency (%) = Biomass (g)/ Total Feed intake (g) x 100
Protein efficiency rate (g) = Total Weight gain of PL (g)/ Total Protein consumed (g)

Energy utilization study

The energy content of whole prawns, feeds, moult and faeces was measured using Parr 1281 Oxygen Bomb Calorimeter. The energy budget was calculated using the equation (C = (P+E) + R + F +U) derived by Petrusewicz & Macfadyen [16]; where, C is the energy consumed in food; P is the growth; R is the material lost as heat due to metabolism; F is the energy lost in faeces; U is the energy lost in excretion and; E is the energy lost in exuvia.

Feeding Rate (FR) = Mean Food Consumption (kcal/day)/Initial live weight of the prawn (g)
Mean Absorption = Mean Food Consumption (kcal/day) – Mean Food Excreted as Faeces (kcal/day)
Absorption Rate (AR) = Mean Absorption (k.cal/day)/ Initial live weight of the prawn (g)
Mean Conversion = Mean weight gain (kcal/day) + Mean exuvial weight (kcal/day)
Conversion rate (CR)= Mean Conversion (kcal/day)/ Initial live weight of the prawn (g)
NH_3 Excretion Rate (ER) = Mean NH_3 Excretion (kcal/day)/Initial live weight of the prawn (g)
Metabolic Rate (MR) = Absorption Rate (kcal/g/day) – Conversion Rate (kcal/g/day) + NH_3 excretion Rate (kcal/g/day)

Biochemical constituents of the experimental animals

The initial and final day of the experiment, the biochemical constituents of the experimental animals were determined. The biochemical constituents, such as total protein [17], amino acid [18], lipid [19], carbohydrate [20], ash and moisture contents [21] of individual diet fed prawns were measured.

Microbial study

Microbial analyses [21] were performed in the experimental PL gut.

Statistical analyses

The data obtained in the present study were subjected to different statistical interpretations. One way analysis of variance (ANOVA; SPSS, 13.0) was used to determine whether significant variation between the treatments existed. Differences between means were determined and compared by *post hoc* multiple comparison test (DMRT). All the tests used a significance level of P<0.05. Data are reported as means ± standard deviations.

1.Mean ± SD
2.One-way ANOVA
3.DMRT

RESULTS AND DISCUSSION

Morphometeric data

Table 2 revealed the morphometeric data of LS+BS (4:3) diets fed PL group. The initial average body length and weight of PL was 1.61±0.05cm and 0.25±0.04g respectively. After the feeding experiment, the growth increment observed was higher in 4% LS+BS supplemented diet fed PL, followed by 3%, 2% and 1% diets when compared with control. These differences were found to be statistically significant (P<0.05). Similar results reported by Seenivasan *et al.* [4] tested different concentrations of *L. sporogenes* bioencapsulated *Artemia* fed *M. rosenbergii* PL had significantly enhanced the final length and weight than the control diet fed PL group. It has been reported by Deeseenthum *et al.* [10] showed that feeding *M. rosenbergii* PL with commercial diets containing 10^7 cfu ml^{-1} of *Bacillus* spp KKU02 and *Bacillus* spp KKU03, enhanced growth performance than control diet fed prawn PL. It has also been reported in rainbow trout, *Oncorhynchus mykiss* fed with *S. cerevisiae* incorporated diets had significantly improved the morphometeric data [22].

Table 2.The morphometric data, growth performance, biochemical constituents and energy utilization of *M. rosenbergii* PL fed with *L. sporogenes+B. subtilis* (4:3) incorporated diets

Parameters	Control	Experimental diets				F-Value
		1% LS+BS	2% LS+BS	3% LS+BS	4% LS+BS	
Initial length cm)	1.61±0.05	1.61±0.05	1.61±0.05	1.61±0.05	1.61± 0.05	-
Final length (cm)	4.72^{d}±0.20	4.92^{cd}±0.27	5.32^{bc}±0.33	5.50^{b}±0.30	6.18^{a}±0.28	12.41
Initial weight (g)	0.25±0.04	0.25±0.04	0.25±0.04	0.25±0.04	0.25±0.04	-
Final weight (g)	1.06^{b}±0.22	1.08^{b}±0.20	1.60^{a}±0.31	1.58^{a}±0.23	2.00^{a}±0.16	8.98
S (%)	82.50±2.50^{b}	80.00±3.00^{b}	80.00±2.50^{b}	82.50±2.50^{b}	87.50±3.00^{a}	3.83
WG (g)	0.81±0.10^{c}	0.83±0.13^{c}	1.35±0.18^{b}	1.33±0.20^{b}	2.07±0.24^{a}	25.21
SGR (%)	0.697±0.023^{b}	0.706±0.035^{b}	0.895±0.026^{a}	0.889±0.029^{a}	1.003±0.210^{a}	5.57
FCR (g)	3.70±0.17^{a}	2.88±0.21^{b}	2.54±0.17^{bc}	2.44±0.23^{c}	2.33±0.19^{c}	24.20
FCE (%)	0.84±0.16^{c}	0.84±0.22^{c}	1.32±0.26^{b}	1.26±0.14^{b}	1.88±0.18^{a}	14.25
PER (g)	0.59±0.07^{d}	0.75±0.03^{c}	0.87±0.05^{b}	0.90±0.04^{b}	1.08±0.06^{a}	38.86
Protein (%)	60.60±3.64^{b}	61.90±2.56^{b}	63.82±2.69^{ab}	65.80±2.46^{ab}	67.60±2.74^{a}	2.97
Amino acid (%)	27.07±3.18^{b}	29.10±2.62^{b}	31.80±3.84^{ab}	33.01±3.19^{ab}	35.80±3.76^{a}	3.09
Carbohydrate (%)	11.60±1.24^{c}	12.04±1.67^{c}	14.08±1.71^{bc}	16.02±1.80^{ab}	17.80±1.28^{a}	8.55
Lipid (%)	7.92±0.71^{b}	9.22±1.59^{b}	10.40±1.42^{b}	13.46±1.38^{a}	14.80±1.68^{a}	12.80
Ash (%)	16.30±1.38^{a}	17.10±1.76^{a}	17.00±1.68^{a}	18.40±1.52^{a}	19.00±1.46^{a}	1.50
Moisture (%)	77.10±3.74^{a}	77.00±3.47^{a}	76.09±3.65^{a}	75.40±3.35^{a}	75.00±3.79^{a}	<1
FR (k.cal/g/day)	0.329±0.061^{c}	0.355±0.087^{bc}	0.393±0.082^{b}	0.459±0.072^{a}	0.505±0.061^{a}	19.19
AR (k.cal/g/day)	0.281±0.058^{d}	0.312±0.071^{cd}	0.354±0.075^{c}	0.424±0.037^{b}	0.475±0.076^{a}	29.35
CR (k.cal/g/day)	0.202±0.064^{c}	0.216±0.083^{bc}	0.239±0.072^{bc}	0.258±0.046^{b}	0.313±0.061^{a}	8.74
AE (k.cal/g/day)	0.011±0.044^{a}	0.013±0.006^{a}	0.014±0.009^{a}	0.014±0.007^{a}	0.019±0.010^{a}	<1
MR (k.cal/g/day)	0.090±0.056^{c}	0.109±0.048^{bc}	0.129±0.062^{b}	0.180±0.052^{a}	0.181±0.036^{a}	15.94

Each value is a mean ± SD of three replicate analysis, within each row means with different superscripts letters are statistically significant P<0.05 (one way ANOVA and subsequently *post hoc* multiple comparison with DMRT).
S: Survival; WG: Weight gain; SGR: Specific growth rate; FCR: Feed conversion ratio; FCE: Feed conversion efficiency; PER: Protein efficiency rate
FR: Feeding rate; AR: Absorption rate; CR: Conversion rate; AE: NH_3 Excretory rate;
MR: Metabolic rate

Survival performance

The survival performance of LS+BS (4:3) diets fed PL group is also given in Table 2. It showed that maximum (87.50%) survival performance was observed in 4% LS+BS incorporated diet fed PL. But it was only 80.00% each in diets 1% and 2% LS+BS supplemented diets fed prawn respectively. Invariably in control and 3% LS+BS diet fed prawns, the survival was similar (82.50%). These differences on survival of control and experimental prawns was found to be statistically significant (P<0.05). Similarly, Seenivasan *et al.* [15] reported that various inclusion levels of Binifit™ (0.5%, 1%, 1.5% and 2%) supplemented diets had the better survival performance in *M. rosenbergii* PL, when compared to the control. Supportively, Saad *et al.* [23] reported that different concentration of Biogen® (1%, 2%, 3% and 4%) incorporated diets had the better survival performance in *M. rosenbergii* PL, than the control. Also, Venkat *et al.* [24] pointed out that the survival performance of *M. rosenbergii* PL fed with bioencapsulated *L. acidophillus* and *L. sporogenes* diets had 100% survival. Fernandez *et al.* [5] reported the enhanced survival rate (92 to 98%) by the probiotics (Lactic acid bacteria) diets fed juveniles of *P. indicus.* Boonthai *et al.* [25] stated that the black tiger shrimp, *P. monodon* fed with probiotic (*Bacillus* sp) supplemented diets was found to have maximum survival rate up to 91.68%.

Growth performance

In this study, LS+BS (4:3) incorporated diets fed prawns resulted in significant increase (P<0.05) of weight gain, specific growth rate, feed conversion efficiency and protein efficiency rate (Table 2). In support to these the FCR was found to decrease (P<0.05) in LS+BS (4:3) incorporated diets fed prawns (Table 2). Therefore, the overall growth was higher particularly, in 4% LS+BS (4:3) incorporated diets fed prawns. This indicates the fact that this much quantity of LS+BS (4:3) addition was required to attain better growth performance in *M. rosenbergii* PL. Similar results have been reported in postlarvae, *M. rosenbergii* fed with *L. sporogenes* supplemented diets [26]. It has been reported that the increase growth performance was achieved by *M. rosenbergii* PL fed with bio-encapsulated diet containing *L. sporogenes* [4]. It has also been reported that Binifit™ supplemented diets have improved the growth performance of the freshwater prawn, *M. rosenbergii* PL [15]. Ranisha *et al.* [6] showed that *M. rosenbergii* fed with probiotic (*Bacillus* spp) supplemented diets had improved the growth performance of PL. Deeseenthum *et al.* [10] reported that *M. rosenbergii* PL fed with *Bacillus* spp KKU02 and *Bacillus* spp KKU03 supplemented diets had significantly increased growth performance than control diet fed prawn PL. Keysami *et al.* [9] pointed out that probioitics *B. subtilis* bio-encapsulated diets had significantly improved the growth performance of the freshwater prawn, *M. rosenbergii* PL. Also, Hisano *et al.* [8] noted that probioitics *Saccharomyces cerevisiae* (2.0%) and yeast derivatives (2.0%) supplemented diets had improved the growth of juvenile *M. amazonicum*. It has also been reported that significantly improved the growth was recorded by *M. rosenbergii* PL fed with bio-encapsulated diet containing *L. acidophilus and L. sporogenes* [24]. Suralikar and Sahu [27] reported that *M. rosenbergii* fed with *L. ceremoris* bio-encapsulated diet had significantly increased the growth performances.

Biochemical constituents

Table 2 also shows the biochemical constituents, such as total protein, amino acid, carbohydrate, lipid, ash and moisture in *M. rosenbergii* PL fed with LS+BS (4:3) incorporated diets. After the feeding trial of 90 days, the levels of these constituents except moisture content was higher (14.80%) in PL fed with 4% LS+BS diet, followed by the PL fed with 3% LS+BS, 2% LS+BS and 1% LS+BS when compared with control diet fed PL group. These differences were found to be statistically significant ($P<0.05$). In the case of moisture content just the reverse was recorded. The decrease in the content of moisture was found to statistically non significant ($P>0.05$) when compared to that of control group. A similar result in proximate biochemical composition was previously observed in *M. rosenbergii* PL fed with *L. sporogenes* supplemented diet has significantly increased the tissues biochemical proximate composition [26]. Seenivasan *et al.* [4] pointed out that probioitics *L. sporogenes* bio-encapsulated diets had significantly improved the tissues biochemical proximate composition of the freshwater prawn, *M. rosenbergii* PL. Also, Seenivasan *et al.* [15] showed that *M. rosenbergii* PL fed with probiotic Binifit™ supplemented diets had significantly increased the tissues biochemical proximate composition. It has been reported in *M. rosenbergii* PL fed with Biogen® supplemented diets had significantly increased the carcasses biochemical proximate composition [23]. Venkat *et al.* [24] noted that probioitics *L. sporogenes* and *L. acidophilus* bio-encapsulated diets had significantly increased the carcasses biochemical proximate composition of *M. rosenbergii* PL.

Energy utilization performance

The results on energy utilization parameters such as feeding rate, absorption rate, conversion rate, NH_3 execratory rate and metabolic rate of LS+BS (4:3) incorporated diet fed group of PL is also proved in Table 2. After the feeding experiment, the energy utilization performance were found to be maximum in prawn PL fed with 4% LS+BS diet, followed by the PL fed with 3% LS+BS, 2% LS+BS and 1% LS+BS diets when compared with control. These differences were found to be statistically significant ($P<0.05$). Similarly, Seenivasan *et al.* [4] reported that probioitics Binifit™ incorporated diets had improved the energy utilization performance of freshwater prawn, *M. rosenbergii* PL. It has been reported that *L. acidophilus* and yeast *S. cerevisiae* supplemented diets have improved the energy budget of Koi Carp, *Cyprinus carpio* [28]. It has also been reported in pearl spot, *Etroplus suratensis* fed with *Lactobacillus* and yeast supplemented diets had significantly improved the feed energy utilization performance [29]. Abdel-Tawwab *et al.* [30] showed that *Saccharomyces cerevisiae* supplemented diets have improved the growth and feed energy utilization performance of Nile tilapia, *Oreochromis niloticus.* It has been reported in Nile tilapia of the nutrient energy utilization performance was higher in Biogen® incorporated diets [31].

Probiotics load in experimental PL gut

In the present study the colony establishment of probiotics such as *L. sporogenes* and *B. subtilis* were found to be higher in PL fed with 4% LS+BS diet, followed by the PL fed with 3% LS+BS, 2% LS+BS and 1% LS+BS diets respectively. These strains were absent in the case of control prawns (Table 3). Similar results have been reported in the gut of *M. rosenbergii* PL fed with bio-encapsulated *L. sporogenes* [4]. It has also been reported in freshwater prawn, *M. rosenbergii* PL fed with bio-encapsulated *L. sporogenes* and *L. acidophilus* that established in the gut [24]. Rengpipat *et al.* [32] reported that the *P. monodon*, fed diets concentration of probiotic *Bacillus* S11 reached mean levels of 10^6 cfu g^{-1} of gut when administered doses in food ranged between 1.39x 10^{10} and 4.69x10^{10} cfu g^{-1}. It has also been reported that the probiotic bacterial colonies established in the intestine of the rainbow trout, *Onchorhynchus mykiss* fed with *Bacillus* spp supplemented diets [33]. Colony establishments like *B. subtilis, L. lactis* and *S. cerevisiae* in *Labeo rohita* [34], *B. subtilis* in the Indian major carps [35], *Lactobacillus* spp in the sea bream, *Sparus aurata* [36], Lactobacil, sporolac, and yeast in Juvenile Goldfish, *Carassius auratus* [37] and *L. acidophilus* and *S.cervisiae* in pearl spot, *Etroplus suratensis* [29] has been reported.

Table 3. Probiotics load in experimental PL gut

Treatment groups	Probiotics	
	L. sporogenes (10^4 cfu cells)	*B. subtilis* (10^4 cfu cells)
Control	-	-
1% LS+BS	24±6	16 ±4
2% LS+BS	37±5	29±8
3% LS+BS	54±3	31±7
4% LS+BS	64±6	48±5

Each value is a mean ± SD of three replicate analyses

The present attempt concluded that the selected probiotics, *L. sporogenes* and *B. subtilis* on combine from at optimized concentrations was found to enhance the survival, growth, tissue biochemical components and energy utilization performance of reared freshwater prawn *M. rosenbergii* PL. Further research on the diets produced with optimized concentration of the chosen probiotic organisms may be evaluated under field condition in the candidate species *M. rosenbergii*.

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