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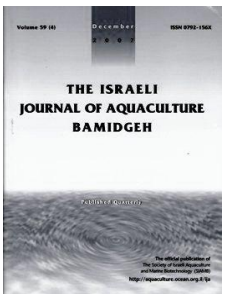

Effect of Supplemented Bacteria (*Lactobacillus sporogenes*) on Growth of *Macrobrachium rosenbergii* Postlarvae

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Abstract

A feeding trial was conducted for 75 days to study the effects of dietary *Lactobacillus sporogenes* on growth and body composition in postlarvae of the giant freshwater prawn, *Macrobrachium rosenbergii* (de Man). Postlarvae were fed one of five experimental feeds containing 0.0% (control diet), 0.1%, 0.2%, 0.5%, or 1.0% *L. sporogenes*. Weight gain, specific growth rate, and protein efficiency ratio tended to increase as the postlarvae were fed the probiotic-supplemented feeds in increasing levels up to 0.5%. The best growth performance was obtained in postlarvae fed 0.5% *L. sporogenes* containing 1.67 x 10^5 colony forming units/100 g feed. When fed 1.0% *L. sporogenes*, growth performance dropped. Tissue protein content was highest in animals fed the 0.5% feed while lipid content was significantly highest in postlarvae fed the control.

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Introduction

Feed is often the major expense in pond production of freshwater prawns *Macrobrachium rosenbergii*, representing as much as 40-60% of the operating costs (D'Abramo and Sheen, 1991). To reduce feed costs and improve performance, feed additives including microorganisms have been tested. Some of the most utilized growth-promoting additives are hormones, antibiotics, ionophores, and salts (Klaenhammer and Kullen, 1999). However, some feed additives, such as antibiotics, can cause intestinal disorders (Hinton et al., 1986) or disease resistance in pathogenic bacteria (Lara-Flores et al., 2003). Residues of antibiotics in aquaculture products may cause problems to human health (WHO, 2006).

Beneficial bacteria are used as alternative feed additives. Probiotics used in aquaculture include photosynthetic bacteria, antagonistic bacteria (*Pseudoalteromonas* sp., *Lavobacterium* sp., *Alteromonas* sp., *Phaeobacter* sp., *Bacillus* sp.), microorganisms for nutritional and enzymatic contribution to the digestion (lactic acid bacteria, yeasts), bacteria for improving water quality (nitrifying bacteria, denitrifiers), and *Bdellovibrio* (Ganguly et al., 2010). The use of combined probiotics (microecologics) is gaining popularity (Wang and Wang, 2008).

To harness the beneficial effects of probiotics, species-specific microorganisms should be isolated and multiplied to facilitate their establishment in the gut of the host fish. However, it is difficult to isolate and multiply indigenous species-specific microorganisms from fish under farm conditions. This study was carried out to evaluate the effect of exogenous *Lactobacillus sporogenes*, a lactic-acid producing bacteria marketed as a probiotic, on the growth performance of *Macrobrachium rosenbergii* (de Man) postlarvae.

Materials and Methods

Culture of postlarvae. Postlarvae from the Central Institute of Fisheries Education (CIFE) hatchery in Mumbai, India, were acclimatized for seven days during which they were fed a control diet and supplied continual aeration from a compressed air pump. After acclimation, postlarvae (92.89±1.27 mg) were stocked in 100-l rectangular tanks at 20 postlarvae/tank. The postlarvae were fed diets containing 0 (control), 0.1%, 0.2%, 0.5%, or 1.0% *Lactobacillus sporogenes* (the weight of the rice bran was reduced as the bacteria content was increased) with three replicates per treatment for 75 days. Equal and sufficient pieces of asbestos sheet and plastic pipe cuttings were provided in every tank as hideouts to minimize cannibalism. Water was exchanged on alternate days. Water temperature, pH, dissolved oxygen, free carbon-dioxide, and carbonate hardness were recorded once a week following methods suggested by APHA (1995). Temperature ranged 26.0-30°C, dissolved oxygen 7.0-7.8 mg/l, and total alkalinity 76.5-128 mg/l. Ammonia (NH_3) ranged 0.07-0.14 mg/l and nitrate-N 0.01-0.08 mg/l. Nitrite-N and PO_4 were within optimum ranges: 0.001-0.006 mg/l and 0.07-0.1 mg/l, respectively.

Feed preparation. For each diet, powdered ingredients were thoroughly mixed with water to make a dough that was steam-cooked 20 min in a pressure cooker (Table 1). The vitamin/mineral premix was added homogeneously after cooling the dough. Bacteria cultures were harvested, washed, and added to the dough together with the vitamin/mineral mix. The bacteria culture was prepared as follows: one gram of lyophilized *L. sporogenes* was suspended in a liter of de Man, Rogosa and Sharpe (MRS) broth (Hi-Media, Mumbai, India), incubated 24 h, and centrifuged at 5000 rpm in a refrigerated centrifuge (Remi, Mumbai, India) to obtain a pellet. The pellet was washed twice with normal saline (0.85%) and weighed. Portions of pellets were added to the feeds to obtain the desired level of bacteria. To determine the number of colony forming units (CFU) of *L. sporogenes* in one gram of feed, 0.1 g of the feed pellet was suspended in 10 ml sterile normal saline (0.85%) and 0.1 ml of this serially diluted sample was plated on MRS agar following the spread plate technique. The dough was pressed in a hand pelletizer to obtain 1.5-mm (diameter) feeds that were dried overnight in a hot air oven at 45°C and stored in air-tight containers at 4°C until use. Every 15 days, the viable CFU per gram feed was determined by a curdling test in which feeds containing *L. sporogenes* were added to sterilized milk (Hugenholtz, 1993). Since the live CFU in the

Table 1. Composition of feed (% dry weight).

	%
Mustard oil cake	20
Rice bran	18.5
Soybean meal	16
Fishmeal	15
Prawn head meal	15
Wheat flour	10
Ragee seed	2
Fish oil	2
Vitamin/mineral mix*	0.5

*Calcium 2.5%, phosphorous 2.5%, potassium 0.4%, salt 0.1%, chloride 0.1%, magnesium 0.15%, iron 3.0 mg, copper 0.1 mg, manganese 0.25 mg, zinc 1.4 mg, vitamin A 1000 IU, vitamin D 100 IU, vitamin E 2 IU, thiamine 0.81 mg, riboflavin 1.0 mg, niacin 10.0 mg, pyridoxine 0.1 mg, vitamin B_{12} 0.5 mg

feeds were reduced by one log cycle after 15 days of storage (measured as the time and temperature that reduced the number by 90%) and by the log of 10 (a little less than two log cycles) after 30 days, feeds were prepared every 15 days.

Biochemical analyses. Proximate compositions of the prawn tissue (carcass) and feeds were analyzed in the Nutrition and Biochemistry Laboratory of the CIFE following standard methods (AOAC, 1995). Moisture was determined by drying weighed samples at 105°C to a constant weight and calculating the difference in weight. Nitrogen contents were analyzed by the Kjeltec system (2200 Kjeltec Auto Distillation, Foss Tecator, Sweden) and crude protein was calculated by multiplying the percent nitrogen by 6.25. Ether extract was designated as crude fat and determined using Soxtec System (1045 Soxtec Extraction Unit, Foss Tecator, Hoganas, Sweden). Diethyl ether (boiling point 40-60°C) was used as a solvent and the weight of the extract was expressed as a percent of the tissue weight. Ash content was estimated by incinerating samples in a muffle furnace at 600°C for 6 h. Total carbohydrate (%) was calculated by subtracting protein, fat, moisture, and ash from the total weight. Proximate carcass compositions were analyzed before initiation and after termination of the experiment.

Screening for lactic acid bacteria. To establish whether the probiotic bacteria integrated into postlarvae organs, tissue samples were weighed, homogenized in sterile saline, and plated on MRS agar and nonselective agar (soybean casein digest agar). Resultant colonies were biochemically analyzed to determine whether any isolate resembled lactic acid bacteria.

Growth study. Postlarvae from each tank were bulk weighed at 15-day intervals to determine weight gain. Growth performance was assessed as percent wt gain = 100(final wt - initial wt)/initial wt, specific growth rate (SGR) = 100(ln final body wt - ln initial body wt)/75 days, feed conversion ratio (FCR) = wt feed given/wt gain, protein efficiency ratio (PER) = wt gain/protein intake, and apparent net protein utilization (ANPU) = 100(final carcass protein - initial carcass protein)/protein fed.

Statistical analysis. Data were statistically analyzed using SPSS version 11.0. One-way analysis of variance (ANOVA) and Duncan's Multiple Range Test (Duncan, 1955) were applied to compare means between treatments.

Results

Gut microbes. Before the start of the feeding trial, eighty bacteria isolates from different postlarvae organs were subjected to biochemical tests recommended for *L. sporogenes*. Colony characteristics and biochemical tests showed that *L. sporogenes* was not present among the indigenous microflora in the postlarvae.

Biochemical composition of feeds and carcasses. Biochemical compositions and bacteria contents of the diets are presented in Table 2. When inoculated in sterilized milk, all probiotic feeds formed a plain curd with a sweet aroma until day 30 after preparation. The control feed did not curdle the media and did not contain *L. sporogenes*. The carcass protein content was higher in postlarvae fed the supplemented feed than in postlarvae fed the control while lipid was highest in the control postlarvae. Moisture was lowest in the control and rose with *L. sporogenes* supplementation while there was no specific trend in ash or carbohydrate contents.

Growth performance. The highest weight gain, SGR, PER, and ANPU and lowest FCR ($p<0.05$) were obtained with the 0.5% diet. There were no significant differences in any growth factors between the 1.0% diet and the unsupplemented control.

Table 2. Proximate composition (means±SD) and viable *Lactobacillus sporogenes* counts of diets; proximate composition and growth of postlarvae (% dry matter basis).

	Level of dietary Lactobacillus sporogenes (%)				
	0 (control)	*0.1*	*0.2*	*0.5*	*1.0*
Proximate composition of feeds					
Crude protein	35.24±0.04	35.23±0.04	35.24±0.03	35.25± 0.02	35.23±0.03
Crude lipid	5.65±0.50	5.66±0.01	5.65±0.01	5.66±0.02	5.63±0.03
Carbohydrate	44.60±0.30	44.6±0.20	44.61±0.10	44.61±0.10	44.58±0.10
Ash	14.49±0.03	14.51±0.03	14.51±0.04	14.52± 0.02	14.52±0.03
Energy (kcal/100 g)	392.94±1.0	392.88±0.64	392.87±0.72	393.03±1.03	392.87±0.67
Lactobacillus sporogenes count in feeds					
Day 0 (x 10^5 CFU/100 g feed)	0	3.31	6.7	1.67	3.35
Day 15 (% decrease)	-	11.94	11.19	12.43	12.09
Day 30 (% decrease)	-	8.96	6.12	5.37	4.63
Proximate composition of carcass					
Moisture	70.98±0.13^{a}	71.32±0.56^{ab}	71.40±0.52^{ab}	71.60±0.52^{ab}	72.14±0.56^{b}
Crude protein	58.58±0.11^{ab}	58.88±0.11^{ab}	58.90±0.09^{ab}	59.03±0.22^{b}	58.72±0.14^{a}
Ether extract	3.32±0.11^{b}	3.24±0.06^{ab}	3.23±0.11^{ab}	3.16±0.07^{ab}	3.09±0.03^{a}
Total carbohydrates	22.23±0.39^{ab}	22.38±0.75^{abc}	21.73±0.13^{a}	22.92±0.16^{bc}	23.02±0.08^{c}
Ash	15.87±0.57^{bc}	15.50±0.66^{abc}	16.13±0.06^{c}	14.88±0.16^{a}	15.16±0.06^{ab}
Organic matter*	84.13±0.57^{ab}	84.50±0.669^{abc}	83.86±0.06^{a}	85.11±0.16^{c}	84.83±0.06^{bc}
Energy (kcal/100 g	355.50±2.82^{ab}	385.87±2.67^{ab}	383.24±0.86^{a}	388.07±0.44^{b}	386.46±0.37^{ab}
Growth parameters					
Initial wt (mg)	92.50±2.5	94.16±1.44	92.50±2.50	91.16±1.25	94.16±1.44
Final wt (mg)	359.37±3.12^{a}	377.08±3.60^{b}	404.16±3.60^{c}	420.83±7.21^{d}	363.54±1.80^{a}
Live wt gain (%)	288.67±9.20^{a}	300.48±5.38^{a}	337.08±8.60^{b}	361.65±9.65^{c}	286.11±5.26^{a}
Specific growth rate (%/day)	1.80±0.03^{ab}	1.84±0.17^{b}	1.96±0.02^{c}	2.03±0.02^{d}	1.80±0.01^{a}
Feed conversion ratio	1.82±0.15^{c}	1.70±0.09^{bc}	1.60±0.05^{ab}	1.48±0.08^{a}	1.81±0.05^{c}
Protein efficiency ratio	1.56±0.13^{a}	1.67±0.09^{ab}	1.76±0.05^{bc}	1.91±0.10^{c}	1.56±0.04^{a}
Apparent net protein utilization	29.57±2.90^{a}	35.42±4.81^{ab}	34.64±2.70^{a}	41.74±3.99^{b}	35.12±2.74^{ab}
Survival (%)	80	80	80	80	80

Values in a row with identical superscript letters do not significantly differ (p<0.05).
*Organic matter = 100 - ash

Discussion

Probiotics have beneficial effects in shrimp aquaculture and commercially available microbes provided through feeds are used to improve growth (Gomezgil, 1995). In this study, *L. sporogenes* improved growth when fed to *M. rosenbergii* postlarvae through prepared feeds up to 0.5% inclusion. The survival of probiotic bacteria in experimental feeds affects growth performance of the host. In the present study, the number of colony forming units in the feeds dropped as the storage period of the feed increased.

The *L. sporogenes*-supplemented feeds improved growth performance in *M. rosenbergii* postlarvae up to an inclusion level of 0.5%. Though the trend of increased weight gain up to 0.5% and subsequent drop at 1.0% may imply that the probiotic effect is best achieved at 0.5%, this can only be analyzed by comparing other parameters such as SGR, FCR, PER, and ANPU. The live weight gain, SGR, FCR, PER, and ANPU of the postlarvae improved up to the level of 0.5% *L. sporogenes*. Similarly, the weight gain, FCR, SGR, and PER significantly improved in *Cirrhinus mrigala* fingerlings when *L. coagulans* and *Saccharomyces cerevisiae* were added to feed (Swain et al., 1996). Survival, growth, FCR, PER, and total heterotrophic microbial count improved in common carp (*Cyprinus carpio*) fed probiotics and spirulina (Ramakrishnan et al., 2008). Growth and survival of *Penaeus indicus* larvae improved when *Lactobacillus plantarum* was added to the rearing medium (Uma et al., 1999). Growth and feed conversion efficiency improved when bio-encapsulated *L. sporogenes* were fed to *M. rosenbergii* through *Artemia* (Venkat et al., 2004). In contrast, weight gain, SGR, and PER fell when the *L. sporogenes* inclusion level was raised to 1.0%. Likewise, percent weight gain was lower

in rohu (*Labeo rohita*) fingerlings fed 0.25% lyophilized *L. acidophilus* than in those fed 0.20% (Ghosh et al., 2004).

The PER and ANPU indicate that supplementing feeds with probiotics significantly improves the protein utilization capacity of *M. rosenbergii* postlarvae. This contributes to optimizing protein use for growth, significant since protein is the most expensive feed nutrient. Protein digestibility improves with the addition of probiotics (Lara-Flores et al., 2003). Better efficiency of protein uptake may be due to better digestion and assimilation of nutrients in the gut by the supplemented micro-flora, possibly by virtue of extra cellular enzymes that play an important role in the digestion process, as observed in turbot larvae (Munilla-Moran et al., 1990).

In conclusion, feeding non-indigenous *L. sporogenes* to *M. rosenbergii* postlarvae through prepared feed improves growth performance. As growth effects are strain and species-specific, further research is needed to investigate the reasons for the reduced growth performance at the higher inclusion level and to determine the most effective dose.

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