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Growth Enhancement and Survival of *Macrobrachium rosenbergii* Larvae Fed *Artemia* nauplii Enriched with Cod Liver Oil and/or *Lactobacillus*

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Abstract

A 60-day experiment was conducted to study the single and combined effects of feeding *Artemia* nauplii enriched with an emulsion containing cod liver oil and/or a probiotic bacteria (*Lactobacillus sporogenes*) on the growth and survival of *Macrobrachium rosenbergii* larvae. *Artemia* enriched with the emulsion (with and without probiotic enrichment) and tissue of *M. rosenbergii* fed such *Artemia* had significantly higher ($p<0.05$) HUFA (20:5 n-3, 22:6 n-3) contents than unenriched *Artemia* or *Artemia* enriched with the probiotic only. The first postlarvae appeared 7-8 days earlier and 95% of the postlarvae 11-12 days earlier in the emulsion-enriched groups than in the control or probiotic-only groups. Growth in the probiotic-only group did not significantly differ ($p>0.05$) from the control. Feeding probiotic-treated *Artemia* reduced pathogenic bacteria (*Vibrio* sp. and *Pseudomonas* sp.) in the gut microflora. The highest survival was recorded in the group fed *Artemia* nauplii enriched with both the probiotic bacteria and the cod liver oil emulsion.

Introduction

The freshwater prawn, *Macrobrachium rosenbergii*, is a tropical species widely distributed in the Indo-Pacific region. Culture of this species is wide spread due to well-established hatchery rearing techniques. Survival during the larvae stage is the main bottleneck limiting the availability of seed. Feed and feeding are the major constraints during larvae rearing. Until recently, live feed was considered best for *M. rosenbergii* larvae and brine shrimp (*Artemia* sp.) was an important live aquaculture feed. However, *Artemia* nauplii are incomplete sources of nutrition because of the lack of highly unsaturated fatty

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acids (HUFA), especially docosahexaoenoic acid (DHA 22:6 n-3; Leger et al., 1986; 1987). The requirement for n-3 HUFA eicosapentaenoic acid (EPA 20:5 n-3) and DHA is critical for larvae during their first feeding.

As a remedial measure, various enrichment techniques using algae that are rich in n-3 HUFA have been tested. Microparticles (Robin et al., 1981) and marine oil emulsions (Watanabe et al., 1982; Leger et al., 1987) improve the essential nutrient contents of *Artemia* nauplii. The filtering of particles (1-50 µm) from the water by *Artemia* nauplii form the basis of the enrichment (Lavens and Sorgeloos, 1996). Supplementation of dietary n-3 HUFA increased growth of *M. rosenbergii* juveniles (Sheen and D'Abramo, 1990) and survival of *Penaeus* and *Macrobrachium* larvae (Bengston et al., 1991). *Macrobrachium rosenbergii* postlarvae fed HUFA-enriched *Artemia* resisted osmotic stress better than control postlarvae (Kontara et al., 1995). *Artemia* nauplii enriched with HUFA-rich cod liver oil improved the metamorphosis of *M. rosenbergii* larvae (Murthy, 1998). Although enrichment diets are commercially available in the form of dried microcapsules, they are relatively expensive and have a limited shelf life, mainly due to lipid auto-oxidation during storage.

Besides nutritional deficiencies, opportunistic pathogenic bacteria are part of the normal microbiota of aquatic organisms and affect survival rates. Larviculture tanks and live food organisms have high microbiotic loads. Studies indicate that *Vibrio* sp. can be transmitted through *Artemia* to larvae (Chair et al., 1994; Sedano et al., 1996), possibly contributing to the poor survival of *M. rosenbergii* larvae during hatchery rearing.

Dietary probiotic supplementation can suppress the bacteria load in fish and shellfish (Bly et al., 1997; Gram et al., 1999). Lactic acid bacteria, being an autochthonous microflora of warm-blooded animals, have often been used as a probiotic species (Gram et al., 1999; Mukherjee and Nayak, 2004). The effects of HUFA or probiotic-enriched *Artemia* nauplii on *M. rosenbergii* were reported by Hemabindu et al. (2004) but no reports are available on the combined effect of HUFA and probiotic supplementation of *Artemia* on *M. rosenbergii* larvae. The present study is designed to assess the potential of polyunsaturated fatty acids (HUFA) and probiotic bacteria enrichment of *Artemia* nauplii on the growth and survival of *M. rosenbergii* larvae.

Materials and Methods

Experimental design. Macrobrachium rosenbergii larvae were equally distributed into 300-l fiber reinforced plastic tanks in a static rearing system with four treatment groups and four replicates of each treatment. One group was fed unenriched *Artemia* (Control), the second probiotic-enriched *Artemia* (Probiotic), the third n-3 emulsion-enriched *Artemia* (Emulsion), and the fourth probiotic and n-3 emulsion enriched *Artemia* (Both) following a completely randomized design for 60 days.

Formulation of the n-3 rich emulsion. The n-3 emulsion was prepared with cod liver oil and water at a ratio of 1:5. The cod liver oil, water, egg yolk, vitamin E, and beta-carotene were blended in a domestic grinder for 90 min. Gelatin was dissolved in warm water and added to the mixture. The mixture was homogenized for 2 min and refrigerated at 4°C for 24 days. The ingredients, proximate analysis, and fatty acid composition of the emulsion appear in Table 1.

Hatching and enrichment of Artemia cysts. Artemia franciscana cysts (Great Salt Lake, UT) were hatched according to Sorgeloos et al. (1986) by incubating them in glass jars at a density of 0.6 g/l for 24 h in saline water (25 ppt) with continuous aeration and light. *Artemia* nauplii were kept in 7 l of water at a density of 100 nauplii/ml in 10-l glass jars and enriched with the above emulsion at 0.5 ml/l and/or a commercially available probiotic strain of *Lactobacillus sporogenes* (7.5×10^7 spores/g; Sporolac, Uni Sankyo Ltd, Mumbai, India) at 10 mg/l. Vigorous aeration was provided throughout the enrichment period to facilitate thorough mixing. At first, *Artemia* nauplii were enriched for 6 h. After 10 days and until the end of the experiment, the enrichment period was prolonged to12 h.

Larvae rearing. *Macrobrachium rosenbergii* larvae (stage 3) were stocked at 140

Table 1. Ingredients and proximate and fatty acid compositions of the n-3 emulsion used to enrich *Artemia nauplii*.

Ingredient	*Quantity*
Water	100 ml
Cod liver oil	20 ml
Egg yolk	11 ml
Gelatin	3.7 g
Vitamin E	40 mg
β carotene	124 mg
Proximate composition	*%*
Protein	3.95
Lipid	16.80
Moisture	78.90
Ash	0.35
Fatty acids	*% of FAME*
14:0	5.1
16:0	20.7
18:0	4.2
18:1 n-9	26.8
18:2 n-6	6.4
20:4 n-6	6.9
20:5 n-3	4.4
22:6 n-3	4.1

per liter in 100 l water at 12 ppt salinity and 27°C. The salinity was gradually reduced to 8 ppt as the larvae began to metamorphose into postlarvae. Water temperature varied 23-25°C. The larvae were fed *Artemia* twice per day (7:00 and 21:00) and egg custard twice per day (11:00 and 16:00) at uniform levels to all groups.

Growth and survival. Growth was expressed as the number of days until the first appearance of postlarvae and the number of days until 95% of the larvae metamorphosed into postlarvae. Postlarvae were counted and removed every two days. Survival was calculated by estimating the difference between the number of postlarvae and the initial number of stocked larvae and expressed as a percentage of the initial number.

Fatty acid analysis. Fatty acid compositions of the emulsion, *Artemia* nauplii, and postlarvae tissue were determined using gas chromatography (fused silica capillary column of 30 m with an internal diameter of 0.25 mm and thickness of 0.20 mm, packed with SP-2330). Lipid was extracted as described by Folch et al. (1957), saponified, and esterified with boron trifluoride methanol (BF_3 methanol) reagent to form fatty acid methyl esters (FAME; AOAC, 1995). The FAME were analyzed using a gas chromatograph equipped with a flame ionization detector. After dilution with 10% chloroform, the samples containing FAME were injected and separated on a fused silica capillary column (as above). The temperature of the injector and detector was maintained at 250°C. The column was operated at a temperature of 180°C for 5 min, then gradually raised to 220°C within 30.64 min with nitrogen as the carrier gas (1 ml/min). Fatty acids were identified by comparing retention time using a standard reference consisting of a mixture of saturated and unsaturated fatty acids. The percentage of area occupied by the different fatty acids was determined for several samples.

Microbiological studies. The viability of the *L. sporogenes* was tested in test tubes containing skimmed milk agar sterilized at 120ºC at 15 psi for 15 min (Hemabindu, 2004). Postlarvae guts (1 g) from each treatment group were thoroughly cleaned and homogenized with sterilized normal saline water. Samples were diluted serially, plated on different media, i.e., MRS agar, nutrient agar, *Pseudomonas* isolation agar, and Thiosulphate Citrate Bile salt Sucrose agar, and incubated as per Hemabindu et al. (2004). Colony forming units (CFU) were counted and expressed as CFU/g.

Statistical analysis. Mean values were analyzed by one-way ANOVA using statistical software (SPSS 11.0 version). Duncan's multiple range test was used to compare means between treatments. Differences were considered significant when $p<0.05$.

Results

Fatty acid contents of Artemia and larvae. The major saturated and unsaturated fatty acids appear in Table 2. There were significantly greater proportions of all unsaturated fatty acids except 18:2 n-6 in *Artemia* nauplii enriched with the n-3 emulsion. The proportion of oleic acid (18:1 n-9) in the *Artemia* nauplii increased significantly due to the enrichment although it did not significantly differ among *M. rosenbergii* larvae. In general, the fatty acid profile of larvae fed the control or probiotic-enriched *Artemia* contained higher proportions of saturated fatty acids and lower proportions of unsaturated fatty acids than larvae fed *Artemia* enriched with the n-3 emulsion.

Microflora in the larvae gut. The number of *Lactobacillus* sp. in the guts of the probiotic-enriched groups was 230 CFU/g while the number was negligible in the control group. The total aerobic bacterial load in the gut of larvae fed the control was 520 x 10^3 CFU/g but only 390 x 10^3 CFU/g in the probiotic-enriched groups (Fig. 1). *Pseudomonas* sp. and *Vibrio* sp. followed a similar trend; there were 110 and 421 CFU/g for the control and

Table 2. Fatty acid contents (% of total FAME) of *Artemia* nauplii and *Macrobrachium rosenbergii* larvae from different experimental groups (n = 4).

Fatty acid	*Control*	*Probiotic*	*Emulsion*	*Both*
Artemia				
Saturated				
14:0	1.4 ± 0.10	1.4 ± 0.09	1.3 ± 0.06	1.2 ± 0.03
16:0	11.3 ± 0.08^b	11.3 ± 0.11^b	11.3 ± 0.04^b	11.7 ± 0.11^a
18:0	6.6 ± 0.07^a	5.9 ± 0.09^b	5.1 ± 0.04^d	5.4 ± 0.10^c
Unsaturated				
18:1 n-9	7.3 ± 0.04^d	18.2 ± 0.09^c	20.1 ± 0.06^b	21.4 ± 0.09^a
18:2 n-6	19.7 ± 0.04	19.3 ± 0.05	19.3 ± 0.07	19.9 ± 0.05
20:4 n-6	1.4 ± 0.03^b	1.5 ± 0.03^b	3.7 ± 0.08^a	3.9 ± 0.10^a
20:5 n-3	0.1 ± 0.01^c	0.1 ± 0.01^c	0.4 ± 0.01^b	0.5 ± 0.03^b
22:6 n-3	not detected	not detected	2.2 ± 0.11^a	2.4 ± 0.10^a
Larvae				
Saturated				
14:0	0.4 ± 0.01^a	0.4 ± 0.01^a	0.2 ± 0.01^c	0.3 ± 0.01^b
16:0	20.0 ± 0.11^a	19.8 ± 0.09^a	11.5 ± 0.05^b	11.7 ± 0.09^b
18:0	16.8 ± 0.04^a	16.8 ± 0.15^a	16.8 ± 0.15^a	11.7 ± 0.13^b
Unsaturated				
18:1 n-9	21.0 ± 0.08	21.1 ± 0.06	20.8 ± 0.09	21.0 ± 0.14
18:2 n-6	4.2 ± 0.05^b	4.1 ± 0.06^b	6.3 ± 0.04^a	6.4 ± 0.11^a
20:4 n-6	1.1 ± 0.04^c	1.2 ± 0.10^c	6.3 ± 0.08^b	8.2 ± 0.05^a
20:5 n-3	1.9 ± 0.07^b	1.9 ± 0.09^b	3.3 ± 0.17^a	3.5 ± 0.24^a
22:6 n-3	0.4 ± 0.03^c	0.4 ± 0.04^c	3.8 ± 0.10^b	4.4 ± 0.09^a

Values in a row with different superscripts differ significantly (p<0.05).

emulsion groups and 34 and 80 CFU/g for the probiotic-enriched groups, respectively.

Growth and survival. Growth and survival of larvae fed the emulsion-enriched *Artemia* were significantly higher than in larvae fed the control or probiotic-enriched *Artemia* (Table 3).

Discussion

The importance of 20:4 n-6 for the growth of fish larvae was demonstrated by Bessonart et al. (1999). Of the unsaturated fatty acids, 20:4 n-6 plays an important role as a precursor of eicosanoids (Castell et al., 1994; Sargent, 1999). Our results support the finding of Han et al. (2000) that the 20:4 n-6 content increased significantly after the 24-h enrichment of *Artemia* nauplii with an emulsion rich in n-3 fatty acids.

The DHA (22:6 n-3) content increased from 0 in the control group to 2.2-2.4% in the emulsion-treated groups. Ando et al. (1999) reported 3.0% and 3.6% increases of 22:6 n-3 in *Artemia* nauplii enriched with fish oil ethyl esters and acetyl-sn-glycerol for 18 and 24 h, respectively. DHA is an essential HUFA for growth of aquatic larvae and is naturally lacking in *Artemia* (Bell et al., 1995). Our study confirmed that HUFA, especially DHA, is deficient in *Artemia*, as faster growth was recorded in the emulsion-treated groups. Lunn and Htoo (1997) reported that the essential fatty acids 18:4 n-6 and 20:4 n-6 were significantly higher (p<0.05) in DHA-enriched *Artemia* compared to newly hatched *Artemia* nauplii.

The saturated fatty acid contents in the control and probiotic larvae groups were significantly higher than those of the emulsion-enriched groups while the reverse was true for n-3 and n-6 fatty acids. This supports the report of Roustaian et al. (1999) that there may be bioconversion of fatty acids to longer chain acids and an increase in the degree of unsaturation in *M. rosenbergii* larvae and postlarvae.

Despite the absence of DHA in the *Artemia* nauplii, larvae in the control and probiotic groups contained DHA, although the lar-

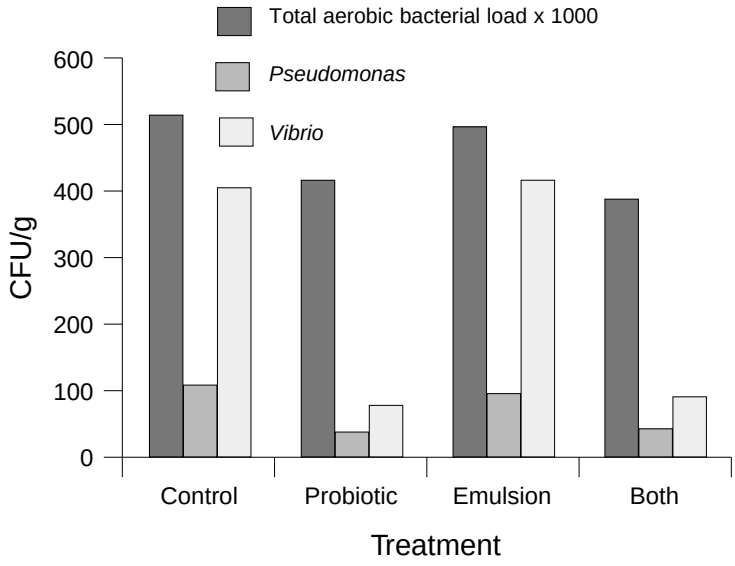

Fig. 1. Microflora in the gut of *Macrobrachium rosenbergii* larvae fed *Artemia* that was unenriched (control), enriched with *Lactobacillus sporogenes* (probiotic), enriched with a n-3 emulsion containing cod liver oil (emulsion), or enriched with both (both).

Table 3. Growth and survival of *Macrobrachium rosenbergii* larvae in different dietary treatments (means±SE).

Treatment	*Appearance of first postlarvae (days)*	*Appearance of 95% postlarvae (days)*	*Survival*
Control	31.0±0.00[a]	56.0±1.00[b]	19.3±0.97[d]
Probiotic	30.6±0.33[a]	55.3±0.33[b]	33.4±0.87[c]
Emulsion	23.6±0.66[b]	45.3±0.33[a]	47.1±1.27[b]
Both	22.6±0.33[b]	44.7±0.33[a]	54.4±0.66[a]

Values in a column with different superscripts are significantly different (p<0.05).

vae fed the emulsion-enriched *Artemia* had higher contents. Apparently, the n-3 enriched *Artemia* nauplii, when fed to *M. rosenbergii* larvae, influences the fatty acid composition, especially the unsaturated fatty acids (20:4 n-6, 20:5 n-3, and 22:6 n-3), of larvae tissues. Roustaian et al. (1999) reported that the fatty acid composition of prawns generally reflects the fatty acid contents of dietary lipids. They also reported on the bioconversion ability of *M. rosenbergii* larvae and postlarvae in chain elongation and desaturation of 16:0, 18:2 n-6, and 18:3 n-3. In addition, they reported that *M. rosenbergii* is capable of converting 16:0 to 18:0 and 18:2 n-6, and 18:3 n-3 to 20:4 n-6 and 20:5 n-3, in agreement with our results.

The significant reduction in the larvae rearing period together with the higher 22:6 n-3 and 20:4 n-6 contents in the larvae tissue suggests that these fatty acids improve larvae growth (Romdhane et al, 1995). Kumlu and Jones (1995) also reported that the number of days until the first appearance of *M. rosenbergii* postlarvae at 27°C was 2-3 days earlier in HUFA treated groups than in the control group. In the present study at 25°C there was an advancement of 7-8 days until the first appearance of postlarvae in the groups fed emulsion-enriched *Artemia*.

The total aerobic bacterial counts were not much affected by the probiotic treatment. But the pathogenic bacteria *Vibrio* and *Pseudomonas* sp. were significantly lower in the probiotic groups, as the probiotic bacteria reduced the substrate for the pathogens in the larvae gut. Gatesoupe (1994) obtained similar results in *Scophthalmus maximus*. The increased survival in the probiotic and emulsion enriched treatment may be due to the DHA enrichment and the suppression of the pathogenic organisms.

There are no parallel reports available on the effect of supplementing both probiotic bacteria and HUFA to *M. rosenbergii* through *Artemia* nauplii. This prima-facie report can be beneficially applied in commercial *M. rosenbergii* hatcheries to improve postlarvae output and overall production by reducing the length of the hatchery phase and the costs of production.

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