The use of two enrichment forms (*Brachionus plicatilis* enrichment and rearing water enrichment) with probiotic bacilli spore on growth and survival of Silver carp (*Hypophthalmichthys molitrix*)

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Summary

This study was carried out to evaluate the effect of probiotic (*Bacillus latrospores* and *Bacillus licheniformis*) on growth and survival of Silver carp larvae (*Hypophthalmichthys molitrix*). Rotifers (*Brachionus plicatilis*) were used as live prey vehicle for probiotic transport. They were cultured in sea water with 15 ppt salinity and *Nannochloropsis oculata* microalgae was used as feed. Five treatments were prepared with three replicates (four experimental treatments and one control). Silver carp larvae were obtained from a local fish farm (Golestan, Iran). Rotifers were filtered from intensive cultivation tanks with 200 rotifer/ml density and then transferred to conical glasses for the enrichment process, and were kept under enrichment conditions for 24 h. Two forms of enrichment were carried out: rotifer biocapsulation and rearing water enrichment. T_1 and T_2 were fed with biocapsulated rotifers and T_3 and T_4 were fed non-biocapsulated rotifers. Instead of biocapsulation, the same density of bacteria was injected directly into the rearing water of T_3 and T_4. T_3 (4.4 ± 1.2 mg) and T_4 (5.05 ± 1.95 mg) treatments of probiotic injected water had significantly higher growth rates than T_1 (2.21 ± 0.94 mg) and T_2 (3.9 ± 0.36 mg), ($P<0.05$). T_1 (3.9 ± 1.03 mg) and T_2 (3.3 ± 0.36 mg) had higher growth rates than the control (2.21 ± 0.94 mg) and were also significantly different from each other ($P<0.05$).

Key words: Rotifer, *B. licheniformis*, *B. latrospores*, Silver carp

Introduction

The word probiotic was first used by Lilly and Stillwell (1965) to mean kinds of organisms which balance bacterial flora in the digestive canal to optimize operation (Jafariyan *et al.*, 2007). Bacteria was at first used to improve human health by decreasing the number of harmful bacteria in the digestive canal (Jafariyan *et al.*, 2007). As knowledge of probiotics improved, scientists began to use bacteria as probiotics for improving the growth rate of animals (Rahimi and Khaksefidi, 2006), especially fishes (Jafariyan *et al.*, 2005). The term

"probiotic" has been defined by Verschuere *et al.* (2000a) as a live microbial adjunct, which has a beneficial effect on the host by modifying the host-associated or ambient microbial community by insuring improved use of the feed or enhancing its nutritional value, by boosting the host's response towards disease, or by improving the quality of its ambient environment (Verschuere *et al.*, 2000a; Planas *et al.*, 2004). Recently, efforts have been made to develop strategies for microbial control in order to decrease the use of therapeutic chemicals and antibiotics (Cabello, 2006). Probiotics, defined by FAO/WHO (2001) as "live microorganisms

which, when administered in adequate amounts, confer a health benefit on the host," constitute a potential tool in the reduction of mortality in the rearing of aquatic organisms (Verschuere *et al*., 2000a; Verschuere *et al*., 2000b; Kesarcodi-Watson *et al*., 2008; Pintado *et al*., 2010). The intensive culture of rotifers by using continuous or periodic additions of selected bacteria has been found to enhance growth and modify the normal microbiota of rotifers (Planas *et al*., 2004). Live prey (rotifers and *Artemia*) are important carriers of contaminated material with their non-selective feeding system to the larval digestive tract (Muroga *et al*., 1987; Blanch *et al*., 1997; Ringo and Birkbeck, 1999), which greatly impacts the microbiota of the larvae and, in many cases, is responsible for significant mortality rates (Muroga *et al*., 1987). Rotifers (*Brachionus* spp.) have been used as a live food for feeding larval marine fishes for over 30 years (Yúfera, 2001). Today, more than 60 marine fish species and 18 crustacean species require adequate and reliable production of high-quality, nutritious rotifers. The success of rotifer mass cultures is determined not only by reproduction rate and density, but also by their nutritional composition and their associated microbiota (Dhert *et al*., 2001; Yúfera, 2001). *Brachionus plicatilis* is the most cultured rotifer in marine fish hatcheries for use as live prey. Similarly, the microalgae *Nannochloropsis oculata* is a commonly-used species for prey cultivation because of its high material composition. The aim of the present study was to evaluate the effect of probiotics with two forms of enrichment on growth and survival of Silver carp larvae cultured in a tropical fish farm.

Materials and Methods

Preparation of microalgae culture (*Nannochloropsis oculata*)

Nannochloropsis oculata is a marine microalgae that is used as the primary feed of choice in commercial hatcheries. *Nannochloropsis oculata* were obtained from *Artemia* Research Center of Uremia University (Iran), and cultivated indoors under laboratory conditions in Erlenmeyer beakers of sterile salt-water (30 g L^{-1}), urea (0.02 g L^{-1}) and phosphate (0.02 g L^{-1}) (Gomishan Shrimp Culture Center, Golestan, Iran). *Nannochloropsis oculata* stock was added into the culture environment. Erlenmeyers were kept at 28°C with heavy aeration. A density of 1×10^4 cell mL^{-1} was achieved.

Rotifer culture

The strain of rotifer used was *Brachionus plicatilis* O. F. Mqller, obtained from *Artemia* Research Center of Uremia University (Iran). Rotifer cultivation was started in tanks ($30 \times 30 \times 35$). Rotifers were fed daily with 200 mL microalgae at a density of 1×10^4 cell mL^{-1}. microalgae (*Nannochloropsis oculata*). This amount of microalgae was increased daily to support rotifers, which were cultured using a batch culture system with 15 g L^{-1} salinity at 25-27°C and medium aeration for oxygen supply. Initially, rotifers were introduced at low density into the tank. The culture strategy consisted of either the maintenance of a constant culture volume with an increasing rotifer density or the maintenance of a constant rotifer density by increasing the culture volume (Qi *et al*., 2009). Rotifers were filtered with 70 µm mesh and washed with 15 g L^{-1} sea water, and then transferred into conical glasses containing 1 liter saltwater (15 g L^{-1}) to be used as food for five Silver carp larvae treatments.

Rotifer biocapsulation

The probiotic species used for enrichment were *Bacillus latrospores* and *Bacillus licheniformis*. Two cones C_1 and C_2 were enriched directly with addition of 1×10^6 CFU mL^{-1} and 2×10^6 CFU mL^{-1} of probiotic, respectively, while C_3, C_4 and $C_{control}$ were not (Table 1). Rotifers in C_1 and

Table 1: Concentration of probiotic used for each cone

Treatment	Control	C_1	C_2	C_3	C_4
Form of enrichment	Without enrichment	Biocapsulation	Biocapsulation	Without enrichment	Without enrichment
Probiotic concentration	0	1×10^6 CFU/L	2×10^6 CFU/L	-	-

C_2 were maintained for 24 h under enrichment conditions (Pintado *et al*., 2010). Then, they were filtered with a 70 µm plankton net and washed with salt water (Pintado *et al*., 2010), and mixed with 1 liter of distilled fresh water.

Silver carp rearing protocols

Silver carp (*Hypophthalmichthys molitrix*) larvae were obtained from a local fish farm and after acclimatization for 5 days in laboratory conditions were divided into 5 treatments (T) (4 experimental treatments and 1 control, replicated 3 times). Three-liter plastic tanks were prepared with aeration and stocked with 30 larvae. For each tank 30% of rearing water was siphoned every day.

Two forms of enrichment were used: rotifer enrichment and rearing water enrichment

Treatments T_1 and T_2 were fed with biocapsulated rotifers from C_1 and C_2, while T_3 and T_4 were fed from non capsulated rotifers cones. The same doses of bacteria were added directly into the larvae rearing water of T_3 and T_4 treatments (Table 2). This process was repeated daily.

Each cone contained 220 rotifers mL^{-1}, and for each larva of Silver carp 366 rotifers were prepared as food. This amount was calculated as 10% of the body weight of Silver carp larvae. Silver carp larvae's initial weight was 0.5 mg and each rotifer was 3.10^{-3} mg. Silver carp larvae were fed twice a day (9 am and 9 pm).

This experiment was run for 14 days, since after this period of time larvae need to feed on larger prey and rotifers are no longer an efficient food source. At the end of the experiment, Silver carp larvae were euthanized by mixing formalin into rearing water, and biometry was performed. Six important growth parameters were calculated, as identified below.
1. Food conversion efficiency (FCE) = [living weight gain (g)/food intake (g)]
2. Food conversion ratio (FCR) = [living weight gain (g)/food intake (g)]
3. Thermal growth coefficient (TGC) = [g final body weight$^{0.333}$ – g initial body weight$^{0.333}$]/ [Water temperature × days of experiment].
4. Weight gain = g final weight of fish – g initial weight of fish
5. Daily growth coefficient (DGC) = 100 × [(final body weight$^{0.333}$ – initial body weight$^{0.333}$)/ days of experiment].
6. Specific growth rate (SGR) = 100 × [l n final weight of fish – l n initial weight of fish)]/days of feeding.

Statistical analysis

The differences in growth rates and parameters among the different experimental treatments were calculated using a one way ANOVA followed by Duncan's multiple range test.

Results

Growth parameters are presented in Table 3. The growth rate of T_4 (5.05 ± 1.95 mg), in which probiotic was added into the rearing water at 2×10^6 CFU/L, was significantly higher than all the other treatments (P<0.05).

The mean weight of T_3, in which probiotic was added into the rearing water at

Table 2: Amount of probiotic used for each treatment

Treatment	Control	T_1	T_2	T_3	T_4
Form of enrichment	Without enrichment	Biocapsulated rotifers	Biocapsulated rotifers	Probiotic added to rearing water	Probiotic added to rearing water
Probiotic concentration	0	1×10^6 CFU/L	2×10^6 CFU/L	1×10^6 CFU/L	2×10^6 CFU/L

Table 3: The performance of length and weight changes

Parameter	Treatment				
	Control	T_1	T_2	T_3	T_4
Mean length (mm)	6.5 ± 0.97^d	7.8 ± 0.8^c	7.5 ± 0.93^{bc}	8.23 ± 1.13^{ab}	8.66 ± 1.21^a
Mean weight (mg)	2.21 ± 0.94^d	3.9 ± 1.03^{bc}	3.3 ± 0.36^c	4.4 ± 1.2^b	5.05 ± 1.95^a

Values in the same row with different superscripts are significantly different (P<0.05)

1×10^6 CFU/L, was significantly lower than in T_4, but significantly higher than those of the biocapsulated groups and the control (P<0.05). Group T_1 (3.9 ± 0.36 mg), which was fed biocapsulated rotifers with a density of 1×10^6 CFU/L of probiotic showed a higher mean weight than T_2 (3.3 ± 0.36 mg) which was fed biocapsulated rotifers with 2×10^6 CFU/L density of probiotic (P<0.05). There was no significant difference between T_2 and T_1 (P>0.05). There was a significant difference between the control and all four treatments. T_4 had the highest growth rate, followed by T_3, T_1, T_2 and the control. There was no significant difference in length among treatments, however, all four were significantly different from the control.

Growth parameters analysis is presented in Table 4 (below).

Food conversion efficiency (FCE) was not different between T_1 and T_2 (P>0.05). The control had the lowest level of FCE. The highest level of FCE was observed in T_4 and there was significant difference between T_4 and T_3. The control had the highest level of food conversion ratio. Thermal growth coefficients (TGC) in T_3 and T_4 were statistically the same (P>0.05). T_2 and T_1 also exhibited no significant difference in TGC. However, there was a significant difference in TGC between control and all treatments (P<0.05). Daily growth coefficient showed the same results as thermal growth coefficient. The specific growth rates (SGR) for T_4 and T_3 were the same and no significant difference was observed between them (P>0.05). Similarly, SGRs of T_1 and T_2 were not significantly different. Significant differences were observed between all experimental treatments and the control. Survival rate analysis showed significant difference

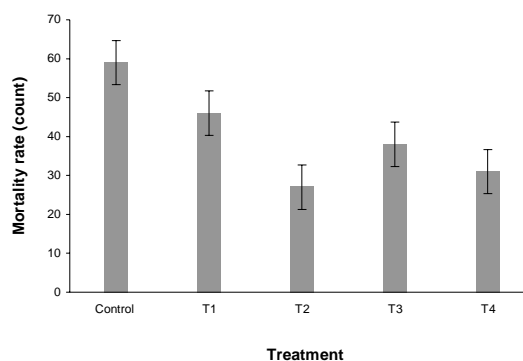between groups, presented in Fig. 1.

Fig. 1: Silver carp larvae mortality rate performance

Discussion

The present study has shown that *Bacillus latrospores* and *Bacillus licheniformis* spores improved growth and survival of *Hypophthalmichthys molitrix*. Different methods of probiotic inoculation in the present study (biocapsulation and probiotic addition into rearing water) revealed that the growth rates of fish in T_1 and T_2 treatments fed with biocapsulated rotifers were statistically similar (P>0.05) but that T_3 and T_4 were statistically different (P<0.05). Addition of probiotic spores into rearing water showed a significant difference, meaning that probiotic spores entered to the larvae gut easily by their activity whether through drinking water or eating feed. Both kinds of enrichment (addition into rearing water and rotifer biocapsulation) showed positive results, and both were significantly different from the control (P<0.05). This positive effect implies that direct addition of probiotic into the rearing water was a significantly better

Table 4: The performance of growth parameter

Parameter	Treatment				
	Control	T_1	T_2	T_3	T_4
FCE	28.7 ± 12.2[d]	50.64 ± 13.41[bc]	42.94 ± 4.7[c]	57.14 ± 16.43[b]	65.67 ± 25.38[a]
FCR	4.29 ± 1.71[a]	2.18 ± 0.64[bc]	2.43 ± 0.31[b]	1.95 ± 0.58[bc]	1.82 ± 0.77[c]
TGC	2.36 ± 4.66[d]	3.13 ± 4.02[bc]	2.92 ± 1.63[c]	3.31 ± 4.49[ab]	3.49 ± 6.38[a]
WG	2.11 ± 0.94[d]	3.8 ± 1.03[bc]	3.2 ± 0.36[c]	4.3 ± 1.26[b]	4.95 ± 1.95[a]
DGC	5.8 ± 1.22[d]	7.81 ± 1.00[bc]	7.28 ± 0.4[c]	8.25 ± 1.12[ab]	8.71 ± 1.59[a]
SGR	21.56 ± 2.79[d]	25.91 ± 1.95[bc]	24.94 ± 0.84[c]	26.73 ± 2.08[ab]	27.48 ± 2.87[a]

Values in the same row with different letter are significantly different (P<0.05). Letters from [a] to [d] represent the highest value to the lowest one

method than biocapsulation (P<0.05). Density of bacteria spore was 1×10^6 CFU/mL^{-1} and 2×10^6 CFU/mL^{-1} of *Bacillus latrospores* and *Bacillus licheniformis*, respectively. *Bacillus* species were obtained from Nikotak Company (Iran) and this density was prepared according to company instructions. Probiotic improved growth rate of Silver carp. A similar study was carried out on Indian carp (*Labeo rohita*) and the same result was observed (Swain *et al.*, 1996). In another study, Bagheri *et al.* (2008) reported that *Bacillus* spp. used as a dietary supplement improved the growth of rainbow trout fry. The difference was not significant for length gain between experimental treatments, but it was significant between experimental treatments and the control (P<0.05). Probiotics reduced mortality rate in experimental treatments and significant differences were observed between treatments and the control (P<0.05). The highest survival rate was that of the T_2 group. A similar dietary addition reduced the mortality rate of Atlantic Cod fry when challenged with *Vibrio anguillarum* (Gildberg *et al.*, 1997). Gomezgil (1995) and Griffith (1995) reported the beneficial effects of nutritional probiotics in developing shrimp of high immunity. Gatesoupe (1994), Robertson *et al.* (2000), Nikoskelainen *et al.* (2001), and Irianto and Austin (2002) reported that the use of probiotic led to higher survival of turbot and Rainbow trout. The control had the highest level of FCR, which implies that rotifers without bacteria have little effect on Silver carp digestion. Low level of FCR showed the best quality of food and this result can be inferred from T_4, which was 1.82 ± 0.779. This study showed that probiotics have a positive effect on fish growth rate, similar to Swain *et al.* (1996), Jafariyan *et al.* (2005), and Jafariyan *et al.* (2007). Jafariyan *et al.* (2007) demonstrated that probiotics had a positive effect on *Huso huso* larvae, with growth rates that decrease FCR from 3.45 to 2.77, implying that digestive operation was improved. Larval growth and survival are affected by the nutritional value of these live feeds, and many studies have been conducted to develop effective methods for enrichment of live feeds with, for example, essential fatty acids and vitamins (Seiji *et*

al., 2009). Gram-positive bacteria, including members of the genus *Bacillus*, secrete a wide range of exon-enzymes (Moriarty, 1998), which may supply digestive enzymes and certain essential nutrients to promote better growth. *Bacillus subtilis* and *B. leicheniformis* can break down proteins and carbohydrates (Rosovitz *et al.*, 1998; Farzanfar, 2006). Therefore, it can be suggested that distribution of *Bacillus* bacteria to trout fry results in enhanced digestion of food and improved growth, including low food conversion ratio (FCR), and high specific growth rate (SGR). Rotifers were reared under enrichment conditions through inoculation of *Bacillus* spp. to rotifer body, and the *Bacillus* spp. probiotic addition were transferred to digestive canal of Silver carp directly. The euryhaline rotifer *B. plicatilis* is a complex species which is commonly used for rearing marine fish larvae. In another study, Jafariyan *et al.* (2009) showed that *Bacillus* species improved digestive tract operation, consistent with our study. T_2, fed with biocapsulated rotifers enriched with 2×10^6 CFU/mL^{-1} density had a lower growth rate than T_1, which was fed with biocapsulated rotifers enriched with 1×10^6 CFU/mL^{-1} density. It is also important to obtain rotifers with higher growth rates and tolerance against environmental stress and better nutritional quality after enrichment (Kesarcodi-Watson *et al.*, 2008). Rotifers were enriched for 24 h and after that filtered with 70 µm mesh, washed and used for feeding (Pintado *et al.*, 2010). This study has shown that the addition of probiotics to the rearing water of Silver carp larvae operates better than the biocapsulation method, as it considerably improves growth rate. Rotifers play an important role as larval starter feed supply. Silver carp is commonly-cultured as commercial fish, and the use of probiotics can improve production and reduce mortality rate.

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plicatilis) and algae (*Nannochloropsis oculata*) samples.

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