Effect of adding probiotics into the rearing tanks of grass carp (*Ctenopharyngodon idella*) for the exploitation of *Artemia urmiana*, *Artemia fransiscana* and *Artemia parthenogenetica* Nauplii

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Key Words:
Artemia; grass carp; nauplii; performance; probiotic.

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Abstract

Bacillus spp. are Gram-positive spore-forming bacteria used commercially as probiotics in the larviculture of fish. *Bacillus* spp. can act positively on cultured organisms by enhancing their survival and growth, stimulating their digestive and immune systems, and improving water quality. In this present study, grass carp larvae were fed on *Artemia urmiana*, *Artemia fransiscana* and *Artemia parthenogenetica* nauplii. In probiotic trials, the combination of *Bacillus circulans* and *Bacillus licheniformis* was also added to rearing tanks of grass carp at 1×10^6 CFU/L. Final body weight, specific growth rate, thermal growth coefficient, daily growth coefficient and relative gain rate were all affected by the addition of probiotic *Bacillus* spp. ($P<0.05$). The greatest improvements in growth parameters were obtained in the group fed *A. parthenogenetica* plus probiotic bacteria, while the group fed *A. urmiana* plus probiotic bacteria had significantly ($P<0.05$) lower growth parameters compared with the other treatments. Thus, the grass carp larvae performed best when fed with *A. parthenogenetica* nauplii and the probiotic bacteria.

Introduction

The use of probiotic *Bacillus* spp. has been suggested as an important strategy to accomplish reproducible outputs in cultivation systems for marine fish larvae and crustaceans (Nogami and Maeda, 1992). The successful colonization by *Bacillus* spp. in the digestive system of larvae involves competition with the established microflora for attachment sites and nutrients. The species composition of the intestinal microflora of fish larvae can be influenced at early stages of development when few, if any, bacteria are present in the larval gut by adding specific bacterial strains to the live food or water (Ringø *et al*., 1996). These probiotic bacteria can increase the activity of digestive enzymes to improve the digestibility of ingested nutrients, and lead to enhancements in growth and feeding performance of fish larvae.

Bacillus spp. are Gram-positive spore-forming bacteria used commercially as probiotics. Due to the physical and biological characteristics of the spore, *Bacillus* spp. preparations have long shelf lives and can be stored in a desiccated form. Moreover, the production costs of such spores for aquaculture are relatively low (Wang *et al*., 2008). *Bacillus* spp. can act positively on cultured organisms by enhancing their survival and growth (Gomez-Gil *et al*., 2000), stimulating their digestive (Ziaei-Nejad *et al*., 2006) and immune systems (Gatesoupe, 1999), and improving water quality as a result of bioremediation (Kennedy *et al*., 1998).

The brine shrimp *Artemia* spp. are common live food organisms used for the rearing of fish larvae. Growth responses of fish to brine shrimp not only depend on the species but also on the geographical strains of *Artemia*. However, determination of the feeding rate on live food such as *Artemia* and the feeding efficiency of fish larvae is very important because the quantity of *Artemia* nauplii fed to cultured fish larvae varies according to species of fish, density and larval developmental stage (Hertrampf and Piedad-Pascul, 2000). Most previous studies have aimed to improve feeding utilization for growth by using different live food sources, including various *Artemia* nauplii. There is no doubt that *Artemia* nauplii is a good live food and energy source and can therefore improve the growth efficiency of grass carp (*Ctenopharyngodon idella*) larvae. Identifying the most suitable *Artemia* nauplii for the grass carp larvae is of major importance for the development of the commercial larviculture of this fish species.

The objectives of this present study were to

investigate the ability of grass carp larvae to exploit *Artemia urmiana*, *Artemia fransiscana* and *Artemia parthenogenetica* nauplii by measuring various fish growth parameters, and to examine the effects of adding of *Bacillus circulans* and *Bacillus licheniformis* to the fish larvae culture medium.

Materials and Methods

Artemia growth and chemical analyses

A. urmiana cysts were donated by the *Artemia* and Aquatic Animals Research Institute of Urmia University. *A. parthenogenetica* was collected from the Urmia and Maharloo Lakes, and *A. fransiscana* cysts were purchased commercially. The chorion of the cysts was chemically removed by decapsulation according to the method of Sorgeloos *et al*. (1977). Decapsulated cysts of *A. urmiana*, *A. fransiscana* and *A. parthenogenetica* were incubated for 24 h in hatching tanks with a conical bottom at 30°C and 30 ppt salinity. The tanks were oxygenated using an electrical air pump and the water surface was constantly illuminated at 2000 lx (Gomez-Gil *et al*., 1998). After 24 h, the newly hatched nauplii were collected in a sieve (pore size of 120 mm) and washed thoroughly with distilled water. Proximate compositions of the nauplii were analyzed using standard procedures described by the Association of Official Analytical Chemists (1990). Moisture was determined by oven drying weighed fresh samples at 100°C for 24 h. Crude protein (nitrogen × 6.25) was determined after acid digestion by micro-Kjeldahl digestion and distillation using a Kjeltec 1026 Distillation Unit together with a Tecator Digestion System (Tecator, Sweden). Lipid was determined by extracting the residue with petroleum ether at 40-60°C for 7-8 h in a Soxhlet apparatus. Ash was determined by ignition at 550°C in a muffle furnace to constant weight.

Preparation of probiotic bacteria

Spores of *B. circulans* and *B. licheniformis* were rehydrated to form vegetative bacteria according to the manufacturer's instructions (Protexin Aquatech, UK).

Fish larvae culture system

Twenty-day old grass carp larvae (mean weight of 120 ± 10 mg) were obtained from Woshmgir Fish Hatchery (Golestan, Iran). Fish larvae were acclimatized to laboratory conditions for 5 d and fed with different *Artemia* nauplii. Each experimental tank was supplied with non-chlorinated water from a deep tube well with continuous aeration. The fish were transferred and randomly allocated in cohorts of 40 to 24 circular fiberglass tanks each of 10 L capacity.

Feeding groups for the fish larvae

In treatments G.P, G.F and G.U the grass carp larvae were fed with *A. parthenogenetica*, *A. fransiscana* and *A. urmiana* nauplii respectively, while in treatments G.P-bacteria, G.F-bacteria and G.U-bacteria the fish larvae were fed with these different species of *Artemia* nauplii, but a blend of two probiotic bacteria (*B. circulans* and *B. licheniformis*) was added to the rearing tanks. Newly hatched nauplii were separated, rinsed and added to the fish tanks four times per day. The feeding rate was 30% of wet body weight per day (Jafryan *et al*., 2009a). The blend of *B. circulans* and *B. licheniformis* was added directly to the tanks at a concentration of 1×10^6 colony forming units (CFU)/L four times a day. The tanks were aerated to keep the live food in suspension, and illuminated by fluorescent tubes (40 W). The water temperature was 24-26°C and the water was replaced four times per day. Each day dead larvae and excessive food were removed from the tanks. The experiment ran for 28 days in total. Samples of water from each tank were collected each day. Serial dilutions of the samples in distilled water and 2.0% (wt/vol) NaCl were plated on tryptic soy agar and incubated at 30°C for 24 h. After 24 h the CFU were counted. At the end of the experiments all the fish were sampled and growth and feeding parameters of the fish larvae were calculated. This experiment was conducted as six distinct trials, each consisting of the six treatment groups performed with four replicates.

Statistical Analysis

The data were analyzed by analysis of variance using SPSS-17 followed by Duncan's multiple range tests.

Results

As shown in Table 1, the compositions of the three *Artemia* spp. differed significantly. *A. urmiana* nauplii contained the greatest quantity of crude protein (56.83%), while the lowest level of crude protein was 39.09%, which was found in *A. parthenogenetica.* The crude lipid contents of *A. urmiana* was the greatest observed (21.20%) while the lipid compositions of *A. fransiscana* and *A. parthenogenetica* were 18.91% and 17.86%, respectively. The crude energy level in *A.*

Table 1. Nutritional composition of the three species of *Artemia* nauplii.

Composition / Species	Crude protein (%)	Crude lipid (%)	Crude Energy (kcal/g)	Dry matter (%)	Moisture (%)	Ash (%)
A. fransiscana	40.65 ± 3.16	18.91 ± 4.2	4673 ± 351	11.76 ± 2.82	88.24 ± 5.66	10.09 ± 1.23
A. urmiana	56.83 ± 6.33	21.2 ± 3.22	4727 ± 245	9.09 ± 1.22	90.91 ± 6.25	3.75 ± 0.20
A. parthenogenetica	39.09 ± 2.33	17.86 ± 1.02	4592 ± 336	11.76 ± 1.12	88.24 ± 6.77	9.53 ± 0.45

Table 2. The values of various growth parameters of the grass carp larvae in different treatment groups (see Material and methods for these groups).

Treatment	G.P-Bacteria	G.F-Bacteria	G.U-Bacteria	G.P	G.F	G.U
IBW (mg)	120 ± 10	120 ± 10	120 ± 10	120 ± 10	120 ± 10	120 ± 10
FBW (mg)	631.5 ± 88.3^{a}	551.8 ± 97.5^{b}	457.7 ± 52.1^{c}	545.3 ± 89.9^{b}	499.1 ± 68.1^{c}	487.8 ± 76.3^{c}
FBL (mm)	42 ± 4.01^{a}	39.87 ± 3.0^{b}	37.65 ± 3.02^{d}	40.43 ± 2.78^{b}	39.49 ± 3.00^{bc}	38.67 ± 2.59^{cd}
SGR[1] (%)	5.80 ± 0.97^{a}	5.36 ± 0.81^{b}	4.67 ± 0.90^{d}	5.34 ± 0.69^{b}	5.00 ± 0.78^{c}	4.94 ± 0.68^{cd}
TGC[2]	0.491 ± 0.10^{a}	0.44 ± 0.08^{b}	0.37 ± 0.08^{d}	0.43 ± 0.07^{b}	0.40 ± 0.07^{c}	0.39 ± 0.06^{cd}
DGC[3]	1.27 ± 0.09^{a}	1.15 ± 0.11^{b}	0.97 ± 0.09^{d}	1.143 ± 0.08^{b}	1.05 ± 0.07^{c}	1.03 ± 0.13^{cd}
RGR[4] (%)	426.2 ± 134^{a}	359.8 ± 100^{b}	281.4 ± 9509^{c}	354.4 ± 86^{b}	315.8 ± 88^{c}	306.5 ± 75^{c}
CF[5]	0.83 ± 0.05^{ab}	0.85 ± 0.06^{a}	0.84 ± 0.14^{ab}	0.81 ± 0.07^{bc}	0.80 ± 134^{c}	0.83 ± 0.05^{ab}
ADG[6] (%)	15.22 ± 2.80^{a}	12.85 ± 1.58^{b}	10.05 ± 2.42^{c}	12.65 ± 1.98^{b}	11.28 ± 2.15^{c}	10.94 ± 1.71^{c}

IBW, initial body weight; FBW, final body weight; FBL, final body length. [1]SGR (specific growth rate) = 100 [ln final weight of fish - ln initial weight of fish)] / days of feeding. [2]TGC (thermal growth coefficient) = [g final body weight$^{0.333}$ - g initial body weight$^{0.333}$] / [water temperature days of experiment]. [3]DGC (daily growth coefficient) = 100 [(final body weight$^{0.333}$ - initial body weight$^{0.333}$) / days of experiment]. [4]RGR (relative gain rate) = 100 [(final weight of fish- initial weight of fish) / initial weight of fish)]. [5]CF (condition factor) = 100 [(g final weight of fish) / (total length of fish in cm)^{3}]. [6]ADG (average daily growth) =100 [(final weight of fish - initial weight of fish) / (initial weight of fish) ×days of feeding].

fransiscana nauplii was 4,673 kcal/g, while *A. parthenogenetica* and *A. urmiana* nauplii contained 4,592 and 4,727 kcal/g, respectively.

The values for the various growth parameters of the grass carp larvae in the different treatment groups are found in Table 2. The growth parameters were significantly affected by the addition of the probiotics to the culture media ($p<0.05$). The *Bacillus* spp. had negative effects on the growth parameters in the G.U-Bacteria group. All the growth parameters observed in the G.P-Bacteria treatment group were significantly different from the other experimental treatment groups ($p<0.05$). The highest condition factor (CF; 0.85) was obtained in the G.F-Bacteria group.

Discussion

The results of this present study indicate that the grass carp larvae had different abilities to exploit the various species of *Artemia* nauplii, and also that the addition of *B. circulans* and *B. licheniformis* to the culturing tank increased the growth rate of grass carp larvae in the G.P-Bacteria and G.F-Bacteria treatment groups. In the G.P, G.F and G.U groups, where the grass carp larvae fed on *A. parthenogenetica*, *A. fransiscana* and *A. urmiana* respectively, the best growth performance was obtained in the G.P group, while the fish larvae in the G.U group showed a reduced ability to exploit the *A. urmiana.* In concurrence with this present study, the beluga (*Huso huso*) showed greatest growth efficiency when fed bioencapsulated *A. urmiana* nauplii with probiotic *Bacillus* (Jafaryan *et al.,* 2007b). Administration of *B. circulans* and *B. licheniformis* via direct inoculation to the rearing tanks resulted in significantly higher fish larvae growth performance compared with the other treatments. Various growth parameters of the grass carp larvae, including final body weight (FBW), final body length (FBL), specific growth rate (SGR), thermal growth coefficient (TGC), daily growth coefficient (DGC), relative gain rate (RGR), CF and average daily growth (ADG) were significantly greater in the G.P-Bacteria and G.F-Bacteria treatments. This is in agreement with the findings of Jafaryan *et al*., (2007a) who also reported higher values of FBW, FBL, SGR and TGC for Persian sturgeon (*Acipenser persicus*) larvae when the suspension of bacillus was used in rearing culture of Persian sturgeon larvae. The RGR and ADG observed for grass carp larvae are similar to values reported for three species of Caspian sturgeon larvae (*Acipenser nudiventris*, *A. persicus* and *H. huso*) (Jafaryan *et al*., 2010) in feeding experiments with bioenriched *Artemia* nauplii and probiotic bacteria. This suggests that the addition of probiotics optimized feed consumption and promoted fish growth parameters (Lara-Flores *et al*., 2003). Similar results were observed by Gatesoupe (1999) using *Bacillus toyoi* in turbot (*Scophthalmus maximus*) and by Swain *et al*. (1996) in Indian carps, in which improved growth factors and feeding efficiencies were recorded. In accordance with the findings in this present study, Jafaryan *et al*. (2010) reported that the use of probiotic *Bacillus* in *A. urmiana* nauplii broth for feeding to *A. nudiventris* larvae had positive effects on fish growth parameters. Moreover, Ziaei-Nejad *et al*. (2006) reported that when probiotic bacilli were added to rearing tanks at 7.3×10^{6} CFU/mL the growth parameters of Indian white shrimp (*Fenneropenaeus indicus*) were increased compared with the control group. Nevertheless, Boyd *et al*. (1984) reported that the addition of commercial probiotic bacteria did not have any significant effects on the growth parameters of channel catfish. Similarly, the addition of bacteria bioencapsulated in *Artemia* metanauplii to a rearing system for halibut larvae (*Hippoglossus hippoglossus* L.) did not increase the growth parameters of this fish species (Makridis *et al*., 2001). These particular bacterial probiotics have considerable extracellular amylolytic, cellulolytic, proteolytic and lipolytic activities (Bairagi *et al*., 2002a), and may reduce anti-nutritional factors such as tannins, phytates and mimosine to minimal values, which reduces the food conversion ratio (Bairagi *et al*., 2002b) and enhances growth performance in culturable fish larvae (Bairagi

et al., 2004). In particular, *B. circulans* is known to produce proteases and other enzymes that enable it to contribute to the natural digestive activities of the host (Ziaei-Nejad *et al*., 2006), and the bacterium itself can also be a source of micro- and macro-elements (Verschuere *et al*., 2000). The best exploitation of *Artemia* was obtained in the G.P-Bacteria group where the grass carp larvae were fed with *A. parthenogenetica* and probiotic bacteria. Nevertheless, the probiotics had negative effects on growth parameters of the grass carp larvae in the G.U-Bacteria group where the fish larvae were fed with *A. urmiana* nauplii and probiotic bacteria. In confirmation of this result, Ghosh *et al*. (2003) highlighted that using *B. circulans* at 1.5×10^4 CFU/g of diet decreased the growth performance of rohu (*Labeo rohita*) larvae. Similar effects were observed by Jafaryan *et al*. (2007b) using probiotic bacilli at 3×10^8 CFU/L in broth of *Artemia* nauplii for feeding to beluga larvae. Ghosh *et al*. (2002) indicated that over concentration of probiotic *Bacillus* reduced the growth parameters of the rohu larvae. It was emphasized that the high specific activity of bacterial extracellular enzymes had negative effects on growth and feeding performance. This suggested that lower concentrations of probiotic *Bacillus* is necessary in rearing tanks of grass carp larvae to give more predictable results of feeding on *A. urmiana* nauplii. Furthermore, adding *B. circulans* and *B. licheniformis* at 1×10^6 CFU/L to the culture system water promoted the consumption of *A. parthenogenetica* and *A. fransiscana* nauplii by the grass carp larvae. This confirmed the results obtained by Carnevali *et al*. (2004), in which *Lactobacillus fructivorans* and *Lactobacillus plantarum* were used for bioencapsulation of *A. fransiscana* nauplii for feeding to sea bream (*Sparus aurata*) larvae. The results of this present study are also in accordance with the findings of Rengpipat *et al*. (2006), in which a probiotic bacilli (*Bacillus S11*) was added into the culture medium of the black tiger shrimp (*Penaeus monodon*), and Gatesoupe (1999) who used *B. toyoi* for feeding turbot (*S. maximus*) larvae. *B. licheniformis* has been shown to act as a growth promoter and it is commonly found in natural fish microflora (Sugita *et al*., 1998). *B. licheniformis* produces extracellular proteases such as amylase and cellulase, which are key enzymes involved in the digestive activities of rohu fingerlings (Ghosh *et al*., 2002). Interestingly, *B. circulans* has been administered with other strains such as *B. licheniformis* to rainbow trout, and this can lead to enhanced growth and improved immune resistance (Raida *et al*., 2003; Bagheri *et al*., 2008).

In conclusion, the present results indicate that the addition of probiotic bacilli to rearing tanks had differential effects on the growth parameters of grass carp larvae when they were fed on different species of *Artemia* nauplii.

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