

### The effects of mixture commercial live bakers' yeast and probiotic bacillus on growth and feeding performance and survival rate of silver carp (*Hypophthalmichthys molitrix*) larvae via bioencapsulated *Artemia urmiana* nauplii

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**Abstract.** *Hypophthalmichthys molitrix* (Valenciennes, 1844) larvae is an important species for freshwater aquaculture. This study evaluated the effects of feeding a blend of probiotic bacilli bacteria (*B. polymixa*, *B. licheniformis*, *B. circulans*) and baker's yeast (*Saccharomyces cerevisiae*) on growth and feeding parameters and survival rate of Silver carp larvae. *Artemia urmiana* (Günther, 1899) nauplii is an important live food that was used as a vector to carry probiotic bacillus to digestive tract of silver carp larvae. The fish larvae were fed at a level of 10 percent body weight at 4 times a day for 30 days. Fish larvae in experimental treatments were fed *A. urmiana* nauplii that were enriched by blend of 50 percent of *Bacillus* spp. with concentrations of  $1 \times 10^5$ ,  $2 \times 10^5$ ,  $3 \times 10^5$  and  $4 \times 10^5$  CFU mL<sup>-1</sup> and 50 percent of baker's yeast with concentrations of  $1 \times 10^5$ ,  $2 \times 10^5$ ,  $3 \times 10^5$  and  $4 \times 10^5$  cells mL<sup>-1</sup> (T1, T2, T3, T4, respectively) and were compared to fish larvae fed control diets of unbioencapsulated *A. urmiana* nauplii. The experiment indicated that feeding and growth parameters in fish fed experimental treatments were significantly higher than fish fed control diets ( $P < 0.05$ ) but survival rate did not significantly differ. Overall, the best group was fed the highest level of yeast and probiotic.

**Key Words:** probiotic, *Saccharomyces cerevisiae*, *Artemia* nauplii, *Hypophthalmichthys molitrix*, bioencapsulation.

**چکیده:** لارو ماهی کپور نقره ای (*Hypophthalmichthys molitrix*, Valenciennes 1844) یکی از مهمترین گونه های پرورشی آب شیرین است. در این مطالعه اثرات مخلوط باسیل های پروبیوتیکی (باسیلوس پلی میکسا، باسیلوس لیشنی فورمیس و باسیلوس سیرکولانس) و مخمر نانوائی (ساکارومایسیس سروبسیا) بر روی کلزایی تغذیه، رشد و نرخ بقا لارو ماهی کپور نقره ای از طریق غنی سازی ناپلی آرتمیا ارزایی شد. ناپلی آرتمیا ارومیان (Günther 1899) یک غذای زنده است که به عنوان حامل برای انتقال باسیل های پروبیوتیکی به دستگاه گوارش لارو ماهی کپور نقره ای استفاده می شود. لاروهای ماهی بر پایه 10 درصد وزن بدن در روز به مدت 30 روز تغذیه شدند. لاروهای ماهی در تیمارهای آزمایشی توسط ناپلی آرتمیا ارومیان که بوسیله  $50$  درصد باسیلوس ها با غلظت  $1 \times 10^5$ ،  $2 \times 10^5$ ،  $3 \times 10^5$  و  $4 \times 10^5$  CFU mL<sup>-1</sup> و 50 درصد مخمر نانوائی با غلظت  $1 \times 10^5$ ،  $2 \times 10^5$ ،  $3 \times 10^5$  و  $4 \times 10^5$  (بترتیب: T1، T2، T3، T4) غنی سازی شده نیز تغذیه شدند، تیمار شاهد با آرتمیای غنی نشده در این آزمایش تغذیه شدند. بعد از 30 روز، آزمایش نشان داد که پارامترهای رشد و تغذیه در تیمارهای آزمایشی بطور معنی داری نسبت به تیمار شاهد افزایش یافته است اما نرخ بقا این تیمارها اختلاف معنی دار زیادی در مقایسه با تیمار شاهد نداشت. نتایج در این مطالعه نشان داد که بهترین تیمار تغذیه ای مربوط به سطوح بالای مخمر و پروبیوتیک بود.

**کلمات کلیدی:** پروبیوتیک، ساکارومایسیس، ناپلی آرتمیا ارومیه، لارو کپور نقره ای، غنی سازی

**Introduction.** Reduced mortality, improved growth and quality of fish larvae are among the beneficial effects that have been obtained by the use of probiotics. This likely occurs through enhanced immunological responses and reduced adherence of pathogenic strains or other modulation of the gut microbiota at specific locations, as has been previously reviewed (Wang et al 2008). Among probiotics, *Bacillus* are gram positive, spore forming bacteria, used commercially as probiotics. *Bacillus* can act positively on cultured organisms by enhancing survival and growth (Gomez-Gil et al 2000). Investigations of the efficiency of *Artemia* nauplii in bioencapsulating bacteria indicate that benefits strongly depend on the type of bacteria used and time of exposure (Patra & Mohamed 2003).

Probiotics are usually defined as live microbial feed supplements, that are administered in such a way as to enter the gastro-intestinal tract which beneficially affects the host animal by improving its intestinal microbial balance and in turn its health (Gatesoupe 1991; Ghosh et al 2003). Bakers' yeast, *Saccharomyces cerevisiae* that is used for the bakers' industry, also can benefit fish growth. It contains various immunostimulating compounds, it has the capability to enhance growth (Oliva-Teles & Gonçalves 2001; Lara-Flores et al 2003; Li & Gatlin 2003, 2004, 2005) on a number of fish species.

*Artemia* bioencapsulation has emerged as a promising method for direct delivery of probiotics to the digestive tract of the target aquaculture species (Gatesoupe 2002; Suzer et al 2008). The investigations indicated that *Artemia urmiana* had a high potential for bioencapsulation with probiotic bacteria to carry their beneficial effects to the digestive tract of cultivable fish larvae (Jafaryan et al 2009a, 2009b).

Previous applications of probiotics have proved beneficial to the host by improving growth, survival and health (Moriarty 1998; Skjeremo & Vadstein 1999).

Thus, this study was designed to evaluate the effects of blend of bacteria and yeast as probiotic on growth parameters and survival rate silver carp larvae via bioencapsulation *Artemia* nauplii.

## Materials and Methods

**Preparation of probiotic *Bacillus* and bakers' yeast.** The probiotic *Bacillus* was prepared from Protexin Co (Iran - Nikotak). The three species of probiotic *Bacillus* bacterial blend under the commercial title of Protexin aquatic were used for bioencapsulation within *Artemia urmiana*. The blends of probiotic bacilli (*B. licheniformis*, *B. circulans* and *B. polymixa*) from a suspension of spores with special media were provided. Four concentrations of bacterial suspension or bacterial broth,  $1 \times 10^5$ ,  $2 \times 10^5$ ,  $3 \times 10^5$  and  $4 \times 10^5$  bacteria per milliliter (CFU mL<sup>-1</sup>) were provided by Protexin Co and the colony forming units (CFU) were assessed by growing in microbial culture in Tryptic Soy Agar (TSA) (Rengpipat et al 1998).

The Baker's yeast (*Saccharomyces cerevisiae*) under the commercial title of Thepax (contain  $1 \times 10^{10}$  cells mg<sup>-1</sup>) was prepared from Doxal Co (Italy). The yeast suspensions or yeast broth were provided with dissolving values of 1, 2, 3 and 4 mg of Thepax powder in 100 mL of distilled water respectively.

**Artemia cyst hatching and bioencapsulation.** The cysts of *Artemia urmiana* from the center of Artemia & Aquatic Animals in Urmia (Iran) were used for this study. The corion of the cysts was chemically removed by employing the encapsulation methodology proposed by Sorgeloos et al (1977). Hatching of the encapsulated cysts was performed in glass container with 1 L of seawater (30 PPT salinity) at a density of 5.0 gram incubated at 30°C with constant illumination and oxygenated via an air pump (Gomez-Gil et al 1998). The bioencapsulation of *Artemia* nauplii was accomplished with density of 2 g live nauplii per liter (Makridis et al 2001). The blends of our bacterial and yeast suspension were with mixed combination of the two concentrations ( $1 \times 10^5$  of *Bacillus* suspension and  $1 \times 10^5$  of yeast suspension) in the treatment of T1; and for the treatments of T2, T3 and T4 with this procedure, the provided suspension of yeast and *Bacillus* were used with concentration of  $2 \times 10^5$ ,  $3 \times 10^5$  and  $4 \times 10^5$  cells mL<sup>-1</sup> in bioencapsulation broth of *Artemia urmiana* nauplii. Our experiment had four treatments and each treatment had three replications.

**Experimental design.** This experiment was conducted in a completely randomized design with five treatments (four of blend bacillus and yeast levels and one control), and three replicates per treatment for a total of fifteen fiberglass tanks (each with capacity of 3 L). Larvae of silver carp were obtained from the Center Hatchery of Shahid Marjanii (Iran). Fish larvae were kept in an indoor fiberglass tank for one week for acclimation to the laboratory conditions. The density of fish larvae was 15 fish larvae per liter ( $10.1 \pm 1.50$  mg,  $3 \pm 0.43$

mm). Silver carp larvae in control and experimental treatments were fed based on the 10 percent of their body weight for 4 times a day (6.00, 12.00, 18.00 and 24.00 H).

The prepared mixture (yeast and *Bacillus*) suspension was added to the bioencapsulation broth of *Artemia nauplii* after 10 h. The bioencapsulated *Artemia urmiana* nauplii were collected on a 120 µm-pore-size sieve, had used for feeding of fish larvae in treatments of T1, T2, T3 and T4 respectively. The control treatment was fed unbioencapsulated *Artemia nauplii*. This experiment was done in 30 days. After each feeding, extra food was collected and deducted from the feed offered. Water quality parameters of input water to rearing system were monitored each week throughout the experimental period. The water average temperature was 26±0.5, pH range was 7.5±0.3 and water dissolved oxygen concentrations ranged from 7.5±0.5 mg L<sup>-1</sup>. During the experiment, an electrical air pump (by a single filtration unit) was used. All the water quality parameters were within the acceptable ranges for fish growth.

**Sample collection.** The fish larvae were weighed individually at the start and at the end of the experiment. At the beginning and at the end of the experiment, 40 fish were seined from each tank and anesthetized with the extract of carnation flower. The fish larvae were weighed by a digital scale with precision of 0.1 mg and total length was measured with a caliper 0.1 mm.

Results were analyzed by a Duncan test from a one-way ANOVA. In All statistical tests, p≤0.05 was taken as the level of significance performed using SPSS 15.0 for Windows.

**Calculation and statistical analysis.** Growth and feeding parameters of fish were calculated based on the data of biometry of silver carp larvae, and included:

Specific growth rate (SGR (%Body weight day<sup>-1</sup>)) = [(Ln BWt<sub>1</sub>-Ln BWt<sub>0</sub>) / t<sub>1</sub>- t<sub>0</sub>] × 100

Specific growth rate (SGR (%Body length day<sup>-1</sup>)) = [(Ln BLt<sub>1</sub>-Ln BLt<sub>0</sub>) / t<sub>1</sub>- t<sub>0</sub>] × 100

Where LnBWt<sub>0</sub> and LnBWt<sub>1</sub> are neperian logarithm of initial and final body weight, LnBLt<sub>0</sub> and LnBLt<sub>1</sub> are neperian logarithm of the initial and final body length of fish larvae and t<sub>1</sub> -t<sub>0</sub> is the sum of experimental days (De Silva & Anderson 1995).

Body weight increase was expressed as: BWI (mg) = BWt<sub>1</sub> - BWt<sub>0</sub> (Tacon 1990); where, BWt<sub>0</sub> and BWt<sub>1</sub> are initial and final body weight of fish larvae, respectively.

Conversion efficiency ratio (CER %) = 100 × (specific growth rate / daily food intake rate) (De Silva & Anderson 1995).

Food conversion ratio (FCR) = 100 × [food intake (g) / living weight gain (g)] (De Silva & Anderson 1995).

Food conversion efficiency (FCE) = [living weight gain (g)/ food intake (g)]×100 (De Silva & Anderson 1995).

Condition factor (CF) = 100× [(g final weight of fish)/ (total length of fish - cm)<sup>3</sup>] (Ai et al 2006).

Thermal growth coefficient (TGC) = [final body weight (g)<sup>0.333</sup>- initial body weight (g)<sup>0.333</sup>] / [Water temperature × days of experiment] (De Silva & Anderson 1995).

Average daily growth (ADG) = 100× [(final weight of fish - initial weight of fish) / (initial weight of fish) × days of feeding] (De Silva & Anderson 1995).

Relative food intake (RFI) = 100× [(feed intake)/0.5] × (final weight of fish - initial weight of fish) × days of feeding] (De Silva & Anderson 1995).

Protein efficiency ratio (PER) = [ living weight gain (g) / protein intake (g)] (Helland et al 1996).

Lipid efficiency ratio (LER) = [living weight gain (g) / lipid intake (g)] (Helland et al 1996).

Total net gain (TNG) = [(final weight of fish(g) - initial weight of fish(g)) × number of fish].

Specific growth (SG) = [3 × (final body weight (g)<sup>0.333</sup> - initial body weight (g)<sup>0.333</sup>) / days of feeding].

Daily feed intake (DFI %) = 100 × [(feed offered (g) - feed collected (g))/ (body weight increase (g) × days of experiment)].

**Results.** The results clearly showed that the mix of *Bacillus* and yeast bioencapsulation had beneficial effects on the growth performance in silver carp larvae. All the probiotic treatments resulted in growth performance better than control ( $p < 0.05$ ) but survival of all groups were not significantly different ( $p < 0.05$ ). However, among the four different concentrations of probiotic fed bioencapsulated in *A. urmiana* to fish larvae, the greatest effects were obtained in treatment 4. This is particularly true for Body weight increased, where the highest was obtained in the experimental treatment T4. Food conversion ratio (FCR) in the experimental treatments was significantly lower than control treatment ( $p < 0.05$ ). Blend of *Bacillus spp.* and yeast had significant positive effects on Specific growth rate (SGR) in experimental treatments in comparison with control. The feeding and growth parameters of silver carp larvae are presented in Table 1.

**Discussion.** The incorporation of probiotics via live food constitutes a very important potential tool for supplying probiotics to the larvae. In the present study, *A. urmiana* was used as a vector to carry the yeast to the digestive system of silver carp larvae. Bacterial colonization of the nauplii could occur externally, via attachment to the body surfaces or internally by ingestion (Gomez-Gil et al 1998). The results of this study clearly demonstrate that the silver carp (*Hypophthalmichthys molitrix*) larvae had different growth and feeding performance in effecting of various concentrations of probiotic bacillus and yeast via bioencapsulation of *Artemia urmiana* nauplii. However, it was seen that the effects of bioencapsulation was dependent on the concentration of the probiotics in the bacterial broth suspension. This was proven by the better fish performance which was observed in the treatment with higher bacterial concentration.

We found that the effects of bioencapsulation depended on the concentration of the probiotics in the broth suspension. Similar results were obtained by Jafaryan et al (2009a) when the *B. polymixa*, *B. circulans* and the baker's yeast were used for sazan (*Cyprinus carpio carpio*) larvae via feeding with bioencapsulated *Artemia urmiana* nauplii. They observed the weight gain and specific growth were significantly increased in probiotic treatments. The maximum of weight gain (242.58 mg) and specific growth (0.346 mg/day) in sazan larvae were obtained when they are feeding by bioencapsulated *Artemia urmiana* in suspension of  $3 \times 10^8$  probiotic bacillus per liter while these parameters in control were 171.91 mg and 0.293 g/day respectively. In our study the best results of weight gain (212.77 mg) and specific growth (0.151mg/day) were obtained in T4, where the the silver carp larvae were fed on bioencapsulated *Artemia urmiana* by  $4 \times 10^5$  CFU/mL.

The similar results in promotion of some growth parameters of turbot (*Scophthalmus maximus*) larvae were observed by Gatesoupe (1991) in using *B. toyoi*.

The survival rate also showed no significant difference between the experimental treatments and control ( $p < 0.05$ ). Final body weight (FBW) in experimental treatments T1, T2 and T4 was significantly higher than control treatment and T3 ( $p < 0.05$ ). These findings demonstrated the ability of probiotics in promotion of growth and feeding performance in fish larvae. The similar effects of *S. cerevisiae* were reported by Jafaryan et al (2009c) in feeding of rainbow trout (*Oncorhynchus mykiss*) larvae by supplemented diet of 4.3 log CFU/g. They indicated that the FCR was decreased from 1.03 in control to 0.92 in probiotic treatment, while the SGR was promoted from 4.24 to 4.61 respectively. A similar result was obtained by Jafaryan et al (2007) when the Persian sturgeon larvae fed on bioencapsulated *Artemia urmiana* nauplii with  $2 \times 10^5$  CFU/ mL of suspension broth. They showed that the best SGR (11.64%), FCR (6.09) and LER (13.72) were obtained in Persian sturgeon larvae fed on bioencapsulated *Daphnia magna* with  $2 \times 10^5$  CFU/ mL of suspension broth while in control these parameters were 10.04, 7.79 and 10.82 respectively.

Similar findings have been reported in other fish larvae including with the dietary supplementation of probiotic *Bacillus spp.* (Bagheri et al 2008) in rainbow trout.

Table 1

The values (mean± SD) of growth parameters of silver carp (*Hypophthalmichthys molitrix*) larvae in experimental treatments (trial 1-4) and control

Treatment	Control	T1	T2	T3	T4
Parameter					
Final body weight (mg)	15.51 <sup>c</sup> ±155.88	26.83 <sup>a</sup> ±200.23	2.61 <sup>ab</sup> ±194.13	21.25 <sup>bc</sup> ±160.92	11.5 <sup>a</sup> ±212.77
Body weight increased (mg)	1.45±0.51 <sup>c</sup>	1.90±0.63 <sup>a</sup>	1.84±0.61 <sup>ab</sup>	1.50±0.47 <sup>c</sup>	2.02±0.51 <sup>a</sup>
Final length (mm)	1.6 <sup>b</sup> ±26.70	3.6 <sup>a</sup> ±35.8	3.9 <sup>ab</sup> ±30.10	0.5 <sup>ab</sup> ±28.7	0.6 <sup>ab</sup> ±31.7
Specific growth rate for weight (% BW/ day)	0.29 <sup>b</sup> ±7.84	0.42 <sup>a</sup> ±8.54	0.03 <sup>a</sup> ±8.47	0.38 <sup>b</sup> ±7.90	0.15 <sup>a</sup> ±8.73
Specific growth rate for length (% BL/ day)	2.18±0.20 <sup>b</sup>	2.46±0.23 <sup>a</sup>	2.30±0.25 <sup>ab</sup>	2.25±0.18 <sup>ab</sup>	2.35±0.20 <sup>ab</sup>
Total net gain (g)	0.31 <sup>c</sup> ±2.91	0.57 <sup>a</sup> ±3.8	0.06 <sup>b</sup> ±3.68	0.43 <sup>bc</sup> ±3.01	0.23 <sup>a</sup> ±4.06
Average daily growth	0.44 <sup>c</sup> ±4.17	0.82 <sup>a</sup> ±5.43	0.07 <sup>ab</sup> ±5.26	0.63 <sup>bc</sup> ±4.31	0.33 <sup>a</sup> ±5.79
Condition factor	408.69±289.23 <sup>a</sup>	591.05±197.01 <sup>a</sup>	645.08±166.6 <sup>a</sup>	561.94±178.02 <sup>a</sup>	671.63±185.91 <sup>a</sup>
Conversion efficiency ratio	25.50±3.21 <sup>b</sup>	36.52±2.09 <sup>a</sup>	33.47±2.28 <sup>a</sup>	27.15±2.12 <sup>b</sup>	37.50±2.35 <sup>a</sup>
Specific growth	0.128±0.007 <sup>b</sup>	0.146±0.011 <sup>a</sup>	0.144±0.001 <sup>a</sup>	0.130±0.009 <sup>b</sup>	0.151±0.004 <sup>a</sup>
Thermal growth coefficient	0.165±0.009 <sup>b</sup>	0.188±0.014 <sup>a</sup>	0.186±0.001 <sup>a</sup>	0.167±0.012 <sup>b</sup>	0.195±0.005 <sup>a</sup>
Food conversion ratio	0.35 <sup>a</sup> ±3.23	0.40 <sup>b</sup> ±2.50	0.04 <sup>b</sup> ±2.54	0.43 <sup>a</sup> ±3.14	0.13 <sup>b</sup> ±2.31
Daily feed intake	8.63±0.89 <sup>a</sup>	6.77±1.04 <sup>b</sup>	6.88±0.93 <sup>b</sup>	8.40±1.10 <sup>a</sup>	6.29±0.33 <sup>b</sup>
Protein efficiency ratio	5.94±0.59 <sup>b</sup>	7.63±1.09 <sup>a</sup>	7.40±0.99 <sup>ab</sup>	6.14±0.83 <sup>ab</sup>	8.11±0.43 <sup>a</sup>
Lipia efficiency ratio	15.86±1.57 <sup>b</sup>	20.37±2.91 <sup>a</sup>	19.75±1.26 <sup>ab</sup>	16.37±2.23 <sup>ab</sup>	21.65±1.17 <sup>a</sup>
Food conversion efficiency (%)	3.31 <sup>b</sup> ±33.30	6.11 <sup>c</sup> ±42.78	0.55 <sup>bc</sup> ±41.48	4.69 <sup>ab</sup> ±34.39	2.46 <sup>c</sup> ±45.46
Survival rate	10.28 <sup>a</sup> ±98.45	6.13 <sup>a</sup> ±99.36	14.78 <sup>a</sup> ±98.64	10.12 <sup>a</sup> ±97.12	17.24 <sup>a</sup> ±99.48

Groups with different alphabetic superscripts differ significantly at  $p < 0.05$  (ANOVA)

Similar results had been reported by Lara-Flores et al (2003); they showed that *S. cerevisiae* improved feeding efficiency of Nile tilapia juveniles, while Noh et al (1994) and Bogut et al (1998) also studied the effect of supplementing carp feeds with different additives. Including antibiotics, yeast (*S. cerevisiae*) and bacteria, they obtained the best growth with a bacterium, not yeast, but their conclusion in carp was in contrast to our results.

The experiment indicated that the blend of *Bacillus* and yeast have the highest ability to increase the growth and feeding parameters in *Hypophthalmichthys molitrix* larvae. However, in trial T4, the silver carp larvae were fed by bioencapsulated *Artemia urmiana* in suspension of  $4 \times 10^5$  CFU/mL, and obtained the best body weight, SGR, PER, LER and TGC, which showed all of them could promote each other. Results of this study also showed that different concentration of yeast could cause different effects on growth parameters.

**Conclusion.** This experiment indicated that using the mixture of probiotic bacillus and yeast could have a great effect on the growth performance of silver carp, and that different levels of the probiotics could have different effects on the growth parameters of the fish larvae.

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