

The Effects on Growth, Survival and Tolerance against Environmental Stressor (High Temperature) of Different Concentrations Probiotic *Bacillus* sp., Fed to Angelfish (*Pterophyllum scalare* Schultz, 1823) Larvae

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Abstract: This study was conducted for 4 weeks to determine the effects of different concentrations probiotic *Bacillus* sp., on angelfish growth, larvae survival rate and tolerance against environmental stressor (high temperature). This experiment was carried out in a completely random design. *Artemia urmiana* naupli was used as a vector to carry probiotic bacillus to digestive tract of *Pterophyllum scalare* larvae. Nauplii with three concentrations of bacteria, 1×10^7 , 2×10^7 and 3×10^7 bacteria mL^{-1} in suspension of broth for 10 h were bioencapsulated and angelfish larvae were fed by them. Angelfish larvae were fed 4 times a day. The control treatment was fed by unbioencapsulated *Artemia nauplii*. At the end of experiment, half of larvae in each treatment were challenged by high temperature (36°C) and their survival was calculated. The results indicated that the probiotic bacillus could influence on growth, survival rate and viability against high temperature stress in angelfish larvae. The final body length in experimental treatments had significant difference in comparison to control ($p < 0.05$). In experimental treatments the survival rate did not show the significant difference ($p > 0.05$) in comparison with control. Nevertheless, viability against high temperature stress in T1 (bioencapsulated *Artemia* with 1×10^7 cfu mL^{-1}) was increased significantly ($p < 0.05$) in comparison with control. The result indicate that different concentrations of bacteria could influence on growth, larvae survival rate and tolerance against environmental stressor (high temperature) in angelfish larvae and the findings can be useful in the performance of larviculture of this species.

Key words: Probiotic, larviculture, viability, angelfish, temperature, Iran

INTRODUCTION

The word probiotic is constructed from the Latin word pro (for) and the Greek word bios (life). The definition of a probiotic differs greatly depending on the source but the 1st generally, accepted definition was proposed by Fuller (1989) as a live microbial feed supplement which beneficially affects the host animal by improving its microbial balance. Animal gut microflora consists of hundreds of different bacterial strains (Walker and Duffy, 1998) able to promote digestion and absorption of nutrients to increase body resistance to infectious diseases (Tannock, 1988) to yield positive effects on growth and to improve general animal welfare (FAO/WHO, 2001). FAO has now designated the use of probiotics as a major means for the improvement of aquatic environmental quality (Subasinghe *et al.*, 2003). In the last decade, the scientific community carefully examined roles and effects of probiotics in aquaculture as an alternative to antimicrobial drugs, demonstrating

positive effects on fish survival (Villamil *et al.*, 2002), growth (Burr *et al.*, 2005), stress resistance (Smith and Davey, 1993; Rollo *et al.*, 2006), immunosystem enhancement (Erickson and Hubbard, 2000; Picchiatti *et al.*, 2007) and finally general welfare (Silvi *et al.*, 2008). Most studies on the effects of probiotics on cultured aquatic animals have emphasized a reduction in mortality or the improved resistance against putative pathogens (Irianto and Austin, 2002). However, the beneficial effects are sometimes temporal, depending on the time of exposure (Verschuere *et al.*, 2000). As most fish contain a specific intestinal microbiota established at the juvenile stage (Olafsen, 2001), the colonization of probiotics to fish intestines requires adequate probiotics presented in ambient Microbial Community (MC) and their interaction with MC should not be neglected. In aquaculture, captive rearing conditions generally can be sources of stress, triggering high mortality, mainly during larval rearing. The use of natural prophylactic supplements in place of chemotherapeutics in aquaculture

has received a great deal of attention in the past decade such preventive products include probiotics. These probiotics can be applied through external bathing or dietary supplementation and have been demonstrated to improve growth performance, feed utilization, digestibility of dietary ingredients, disease resistance and stimulate the immune response of aquatic animals (Gatesoupe, 2008; Kesarcodi-Watson *et al.*, 2008; Wang *et al.*, 2008; Merrifield *et al.*, 2010). Aquatic probiotics have been defined as live microbial supplements that can modulate microbial communities and improve microbial balance, thus providing benefits to the host (Gram *et al.*, 1999). Furthermore, probiotic *Bacillus* species have been shown to improve digestive enzyme activities, growth and survival of crustaceans (Liu *et al.*, 2009; Rengpipat *et al.*, 1998, 2003; Wang, 2007; Zhou *et al.*, 2009). The beneficial effects of these probiotics include higher growth and feed efficiency, prevention of intestinal disorders and pre-digestion of anti-nutritional factors present in the ingredients. Also, existing literature on probiotics usually focused on resistance to some aquatic pathogens such as *Vibrio* sp. (Villamil *et al.*, 2003; Planas *et al.*, 2006), *Amyloodinium ocellatum* (Li *et al.*, 2005) and *Carnobacterium* sp. These studies mainly focused on effects of probiotics on enhancement of survival and nutritional parameters such as feed efficiency and feed conversion ratio. *Bacillus* can act positively on cultured organisms by enhancing survival and growth (Gomez-Gil *et al.*, 2000) by stimulating the digestive (Ziaei-Nejad *et al.*, 2006) and immune systems (Gatesoupe, 1999) and by improving water quality in terms of bioremediation (Kennedy *et al.*, 1998; Moriarty, 1998). Several studies demonstrated these positive effects using a single or two probiotic strains and just few studies described the effects of a mixture of probiotics in fish and shrimp aquaculture (Balcazar, 2003; Lara-Flores *et al.*, 2003; Ziaei-Nejad *et al.*, 2006). Concurrently, *Bacillus* species can be found in marine environment and are part of the microflora of several marine species (Kennedy *et al.*, 1998; Hovda *et al.*, 2007). Little studies had been carried out to incorporate probiotics into a freshwater species common carp, *C. carpio* (Yanbo and Zirong, 2006) and crustaceans, Indian white shrimp *Fenneropenaeus indicus* (Ziaei-Nejad *et al.*, 2006) and shrimp *Penaeus vannamei* (Wang, 2007) based on growth performances and digestive enzyme activities. As stated above, the application of probiotics in aquaculture as the environment friendly treatments has been also increasing rapidly (Gatesoupe, 1999, 2002, 2007) and some papers were associated with the effect of probiotics in fish and other marine organisms (Mohanty *et al.*, 1993; Sharma and Bhukhar, 2000). Probiotics is usually defined as live

microbial feed supplements that are administered in such a way as to enter the gastrointestinal tract and to be kept alive; this beneficially affects the host animal by improving its intestinal microbial balance and in turn its health (Gatesoupe, 1999, 2007). As described by several researchers, probiotics in aquaculture have been demonstrated to have several modes of action: competitive exclusion of pathogenic bacteria through the production of inhibitory compounds; improvement of water quality; enhancement of immune response of host species and enhancement of nutrition of host species through the production of supplemental digestive enzymes (Thompson *et al.*, 1999; Verschuere *et al.*, 2000). The brine shrimp *Artemia* sp. are common live food organisms used for the rearing of marine fish larvae. These have been considered as possible vectors for the delivery of different substances such as nutrients and probiotics (Gatesoupe, 1991). This positive effect of probiotics may be attributed to their ability to outcompete other bacteria (Austin *et al.*, 1995) or to produce micronutrients important for the development of fish larvae (Ringo *et al.*, 1992). Several bacteria have been used as probiotics in the larval culture of aquatic organisms and they can be either delivered directly into the water or via live carrier such as *Artemia nauplii* and rotifers or else added to pelleted dry food (Gomez-Gil *et al.*, 2000). The aim of this study was to evaluate the effects of probiotic bacillus on the growth, larvae survival rate and tolerance against environmental stressor (high temperature) in angelfish larvae.

MATERIALS AND METHODS

Preparing of probiotic bacillus: The probiotic bacillus was prepared from Protexin Co. (Iran-Nikotak). The five species of probiotic bacillus as bacterial blend under the commercial title of Protexin aquatic were used for bioencapsulation of *A. urmiana*. The blends of probiotic bacilli (*Bacillus licheniformis*, *Bacillus subtilis*, *Bacillus polymixa*, *Bacillus laterosporus* and *Bacillus circulans*) from suspension of spores with special media were provided. Three concentrations of bacterial suspension, 1×10^7 , 2×10^7 and 3×10^7 bacteria mL^{-1} (cfu mL^{-1}) were provided by Protexin Co. and the Colony Forming Unit (CFU) of probiotic bacilli were tested by microbial culture in Tryptic Soy Agar (TSA) (Rengpipat *et al.*, 1998).

Artemia cyst hatching and bioencapsulation: The cysts of *A. urmiana* from the center of Artemia and Aquatic Animals in Urmia (Iran) were used for this study. The corions of the cysts were removed chemically by using the methodology that proposed by Sorgeloos *et al.*

(1977). This process is known as decapsulation. Hatching of the decapsulated cysts was performed in glass cone with 1 L of seawater (3.0‰ salinity) at a density of 5.0 g L⁻¹ and incubated at 30°C with constant illumination and aeration through setting air pump (Gomez-Gil *et al.*, 1998). The bioencapsulation of *Artemia nauplii* were accomplished with density of 2 g live nauplii L⁻¹ (Makridis *et al.*, 2001) for 10 h and with three concentration of 1×10⁷ (T1), 2×10⁷ (T2) and 3×10⁷ (T3) bacteria mL⁻¹ in suspension of broth.

Experimental design: The experiments were conducted from June to August 2010 in Institute of Ornamental Fish Hatchery in Babol, Iran. After yolk sac absorption, larvae were divided to twelve glass aquaria (80×30×40 cm) with three replicates for experimental and control treatments. This experiment was conducted in a completely randomized design with four treatments (treatment 1-3 and control).

The density of fish larvae in per tank were 10 fish L⁻¹. Angelfish larvae in control and experimental treatments were fed 4 times a day (8.00, 12.00, 16.00 and 20.00). The control treatment was fed unbioencapsulated *Artemia nauplii*. Gentle aeration was provided by air stones. During the experiment, the water quality parameters were monitored during the trial and average value for temperature, dissolved oxygen, hydrogen ion concentration (pH) and salinity were 26±2°C, 5.7-7.7 mg L⁻¹, 6.9-7.8 units and 0.1 mg L⁻¹, respectively. Dark cycle of 12:12 h was maintained during the experiment. Angelfish larvae were reared for 30 days. Fish from each aquarium were counted and their length was measured to monitor growth and mortalities were recorded.

High temperature challenge: At the end of experiment for evaluation of the larval quality, half of the larvae (in each replicate) were challenged by high temperature. In this propose, larvae were transferred to other aquariums and temperature was increased to 36°C (10°C higher than cultivation temperature) and survival duration was calculated.

Calculations and statistical analysis: The following variable was calculated (Ai *et al.*, 2006):

$$\text{Survival} = N_t \times 100 N_0^{-1}$$

N_t and N₀ were final and initial numbers of larvae in each replicate, respectively and t is the experimental period in days. Results are presented as means±SD. Significant differences among treatments were determined

by Analysis of Variance (ANOVA) and the differences between means were tested with Duncan's multiple-range test using SPSS 16.0 programme. Differences were considered significant at p<0.05.

RESULTS AND DISCUSSION

Effect of probiotic bacillus on growth and viability: Among the three different concentration of probiotic bacillus in bioencapsulation of *Artemia nauplii* which was fed by angelfish larvae, the highest results obtained in T1 (bioencapsulated *Artemia* with 1×10⁷ cfu mL⁻¹). As shown in the Fig. 1, growth of angelfish larvae was significantly (p<0.05) affected by probiotic bacillus. In the experimental treatments, growth was significantly (p<0.05) higher than control treatment. The highest average of length was obtained in the experimental treatment of T1 however, there was no significant difference among experimental treatments (p>0.05). The survival rate of angelfish larvae at the end of experiment is showed in Fig. 2. The significant difference was not observed in experimental treatments in comparison with control (p>0.05).

Effect of probiotic bacillus on larval tolerance: As shown in the Fig. 3, differences in larval tolerance against high

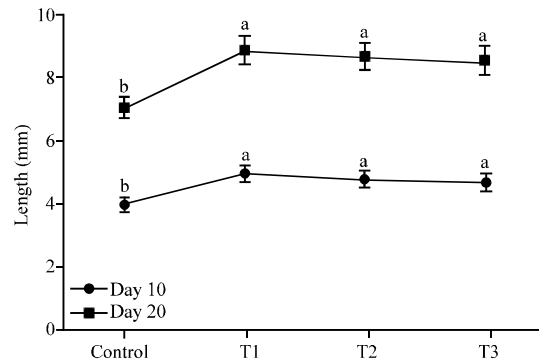


Fig. 1: Growth rate in experimental groups after 10 and 20 days

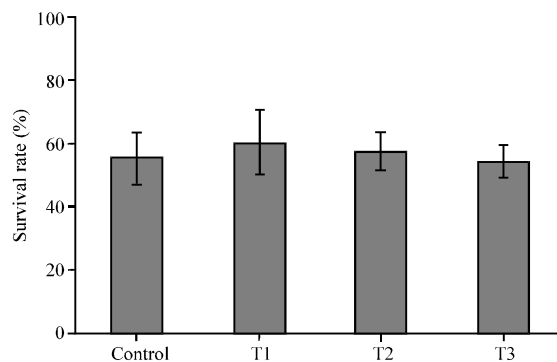


Fig. 2: Survival rate (%) of larvae in day 20th

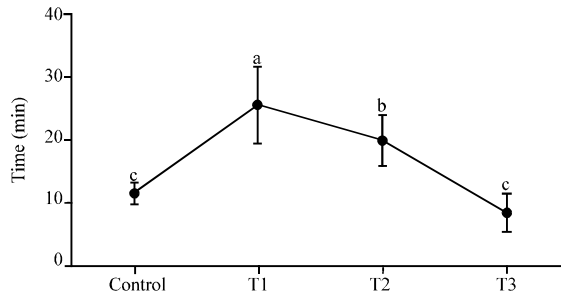


Fig. 3: Larval viability against high temperature stress

temperature stress (36°C) were observed among experimental groups and they were significant among them. The highest and lowest times of survival in 36°C were observed in T1 and T3, respectively.

The current study demonstrates different concentrations of bacteria for enrichment *Artemia* with commercial *Bacillus* sp. was significantly difference in growth between experimental treatments in comparison with control. Similar findings have previously been documented in preliminary trials on larval *H. gammarus* (Daniels *et al.*, 2006). Also, this result indicates that different concentrations of bacteria not differed significantly in survival. Since, the 1st use of probiotics in aquaculture, a growing number of studies have demonstrated their ability to control potential pathogens and to increase the growth rates and welfare of farmed aquatic animals (Gatesoupe, 1991; Lara-Flores *et al.*, 2003; Carnevali *et al.*, 2004; Macey and Coyne, 2005; Wang *et al.*, 2005; Yanbo and Zirong, 2006). Here, we report for the 1st time, an enhancement of the growth rate of the *Pterophyllum scalare*, one of the most important ornamental species in the Iran. The positive effect of *Bacillus* were observed by Gatesoupe (1991) in using *Bacillus toyoi* on turbot (*Scophthalmus maximus* Linnaeus, 1758), Swain *et al.* (1996) in Indian carps that improved the growth factors and feeding performance and Ghosh *et al.* (2003) on the Rohu. Noh *et al.* (1994) and Bogut *et al.* (1998) showed that a commercial probiotic preparation of *Streptococcus faecium* improved the growth and feed efficiency of carp (*Cyprinus carpio*).

Bagheri *et al.* (2008) found that supplementation of trout starter diet with the proper density of commercial *Bacillus* probiotic could be beneficial for growth and survival of rainbow trout fry. Ghosh *et al.* (2002) indicated that the *B. circulans*, *B. subtilis* and *Bacillus pumilus*, isolated from the gut of Rohu have extracellular protease, amylase and cellulose and play an important role in the nutrition of Rohu fingerlings. The photosynthetic bacteria and *Bacillus* sp. (isolated from the pond of common carp) was used in diet of common carp (*Cyprinus carpio* Linnaeus, 1758) by Yanbo and Zirong (2006). It is

important to consider the possibility of using different species as suggested by Noh *et al.* (1994) and Bogut *et al.* (1998). Here, we studied the effects of a combination of *Bacillus* sp. (*Bacillus licheniformis*, *Bacillus subtilis*, *Bacillus polymixa*, *Bacillus laterosporus* and *Bacillus circulans*) at different concentrations of enrichment on growth and survival in angelfish larvae. The results indicated that always growth and survival rate do not increase with increasing concentrations of probiotics, similar results were reported by Jafaryan *et al.* (2010).

Cavalli *et al.* (2003) evaluated the effect of dietary supplementation of vitamins C and E on maternal performance and larval quality of the prawn *Macrobrachium rosenbergii*. They tested the tolerance of newly hatched and 8 days old larvae of *M. rosenbergii* to ammonia exposure. Their results shown newly hatched and 8 days old larvae tolerance tended to increase with increasing levels of AA and higher dietary levels of α -tocopherol acetate did not affect the tolerance to ammonia of newly hatched larvae but it positively augmented the ammonia tolerance of 8 days old larvae.

The study documented that larval survival duration against high temperature stress was promoted in T1 and T2 but resistance against stress in T3 was similar to control group. As a result larvae tolerance tended to decrease with increasing levels of probiotic.

CONCLUSION

In this study, this experiment indicated that the probiotic bacillus have the highest ability to promote the growth in *Pterophyllum scalare* larvae. Different concentrations of probiotic bacilluse had the same effects on the growth in angelfish larvae but the best survival duration against environmental steressor (high temperature) was obtained in T1 (bioencapsulated *Artemia* with 1×10^7 cfu mL⁻¹). In general, the findings can be useful in the performance of larviculture of this species.

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