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Article in *Journal of Applied Aquaculture* · August 2017

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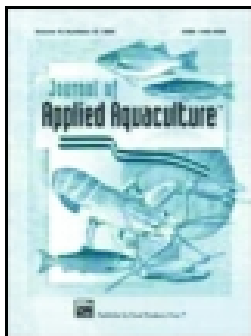
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To cite this article: Fateme Taridashti, Khayyam Delafkar, Amin Zare & Ghobad Azari-Takami (2017): Effects of probiotic *Pediococcus acidilactici* on growth performance, survival rate, and stress resistance of Persian sturgeon (*Acipenser persicus*), Journal of Applied Aquaculture, DOI: [10.1080/10454438.2017.1363112](https://doi.org/10.1080/10454438.2017.1363112)

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Accepted author version posted online: 04 Aug 2017.
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Effects of probiotic *Pediococcus acidilactici* on growth performance, survival rate, and stress resistance of Persian sturgeon (*Acipenser persicus*)

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ABSTRACT

This study was conducted to examine the effect of *Artemia urmiana* nauplii enriched with *Pediococcus acidilactici* on growth performances, survival rate, and stress resistance of Persian sturgeon (*Acipenser persicus*). *Artemia* nauplii were enriched with *P. acidilactici* at concentration of 10^{10} CFU/ml for 3 h (T3), 6 h (T6), 9 h (T9), and one nonenriched nauplii treatment (control). Since nauplii enriched for 9 h had the most significant CFU/g, fish were fed with T9 for 11 days and compared with the control. To evaluate the effect of probiotic on fish resistance, Persian sturgeon larvae were subjected to osmotic shocks of 15, 25, or 35 ppt; four pH treatments, pH 5, pH 6, pH 8 or pH = 9; and air for 5, 10, 15, or 20 s on the 11th day. There were no significant differences in final weight, weight gain, CF, SGR, and FCR between T9 and control ($P > 0.05$). However, the survival rate of larvae fed nauplii enriched for 9 h increased significantly in comparison with the control group ($P < 0.05$). In addition, significant higher resistance to all stress challenges was observed in the T9 group.

KEYWORDS

Artemia nauplii; enrichment; lactic acid bacteria; probiotics; stress tolerance

Introduction

Persian sturgeon (*Acipenser persicus*) is of high commercial interest in several countries thanks to its meat and roe, which is eaten as caviar (Carmona et al. 2009). However, overfishing, loss of spawning grounds, and environmental pollutions resulted in depletion of the stock of this species; according to the International Union for Conservation of Nature (IUCN), the Persian sturgeon is under severe threat of reduction of natural population and extinction (Bronzi et al. 2011). Hence, efforts are deployed by the sturgeon hatcheries for stock restoration and rehabilitation (Mohseni et al. 2008; Williot et al. 2001).

Among the major problems related with the restocking of Persian sturgeon are the low rate of larval survival (Suboski and Templeton 1989) and dramatic level of mortality of newly released individuals (Olla et al. 1998;

Suboski and Templeton 1989). Thus, it is crucial to assess the stress resistance and survival of sturgeon juveniles during the primary stages of life to potentially improve the success of restocking initiatives.

The use of probiotics has opened a new era in aquaculture science, and probiotics are considered health-promoting foods that enhance growth performance, survival, and health status in farmed fish (Kiron 2012; Lauzon et al. 2014; Merrifield et al. 2010; Ringø et al. 2010). Probiotics were initially defined as a live microbial feed supplement that beneficially affects the host animal by improving its intestinal balance (Fuller 1989). However, this definition is being refined (Farzanfar 2006; Gram and Ringø 2005; Spanggaard et al. 2001; Verschuere et al. 2000). According to these authors, “probiotic for aquaculture is a dead, live or component of a microbial cell that, when administrated via the rearing water or the feed, benefits the host by improving either growth performance, disease resistance, health status, feed utilization, or stress response, which is achieved through improving the environmental microbial balance of the hosts.” Probiotics have been demonstrated to improve stress tolerance in some studies, to prepare fish to face critical operations like transport, changing temperatures, periodic manipulation, or any other stressful situations (Varela et al. 2010).

Artemia nauplii are one of the most important live feeds for commercial production of fish larvae in marine and freshwater aquaculture (Jalali et al. 2009). Considering the nonselective and continuous feeding of *Artemia*, it feeds upon a wide variety of food particles (Seenivasan et al. 2012). Therefore it has been used as a live feed and a multipurpose vector for the delivery of different materials in aquaculture such as essential nutrients (Watanabe et al. 1983), antimicrobial agents (Dixon et al. 1995), vaccines (Campbell et al. 1993), and probiotics (Gatesoupe 1994). The enrichment of *Artemia* nauplii with probiotics bacteria is associated with a positive effect on host organisms by improving the performance of the microbiome (Parta and Mohamed 2003). In fish, the lactic acid bacterium (LAB) was described as part of the normal microbiota (Ringø and Gatesoupe 1998; Robertson et al. 2000). It has been reported to improve water quality, fish growth, and survival, which in turn ultimately increases aquaculture output (EL-Haroun et al., 2006; Gatesoupe 1994; Jafariyan et al., 2015; Planas et al. 2004; Shiri Hanzevili et al. 1998; Skjermo and Vadstein 1999; Talpur et al. 2014; Varela et al. 2010; Verschurere et al., 2000), and in some cases it inhibits the growth of pathogenic bacteria (Gilderg et al., 1997). Different studies have examined the effects of probiotics on sturgeon growth performance, health conditions, and physiology (Faramarzi et al. 2011, Faramarzi, Jafaryan, Roozbehfar, Jafari, and Biria 2012; Faramarzi, Jafaryan, Roozbehfar, Jafari, Rashidi, and Biria 2012; Iranshahi et al. 2011; Jafaryan and Soltani 2012); however a review of the literature revealed that documentation on the effects of probiotics on Persian sturgeon larvae is limited. Hence, this study was conducted to investigate the effects of the enrichment of *Artemia urmiana* with *Pediococcus acidilactici* on growth, survival, and stress resistance in Persian sturgeon.

Materials and methods

Probiotic and Artemia nauplii enrichment

The dried powder of Bactocell (Lallemand Co., Montreal, Canada), which is a food additive that contains 10^{10} CFU ml⁻¹ of *Pediococcus acidilactici*, was used as a probiotic. *Artemia urmiana* (Urmia Lake, North West of Iran) cysts were decapsulated with sodium hypochloride prior to hatching (Hoff and Snell 1987). Cysts were allowed to hatch in a conical tank (40 L volume) containing 33 ppt salinity seawater over a period of 24 h, at 28°C, under 2000 Lux illumination and aeration. Freshly hatched nauplii were reared for 24 h up to the instar-II stage. The instar-II *Artemia* were harvested by exploiting the positive phototaxis behavior of nauplii. Instar-II *Artemia* nauplii were transferred into glass containers with 500 ml water volume and stocked at the rate of 100 nauplii per ml. The three different *Artemia* enrichment treatments consisted of feeding nauplii with *P. acidilactici* at final concentration of 10^{10} CFU/ml for 3 h (T3), 6 h (T6), and 9 h (T9), while there was no enrichment in the control treatment (nonenriched *Artemia*). During the enrichment, water temperature was maintained at 15°C, and the water was aerated. Each enrichment treatment was done in triplicate. After the 3 h enrichment, 10 ml were sampled from each replicate of control and T3 to determine bacterial load and transferred into sterilized experiment tubes for bacteriological sampling. This procedure was repeated for T6 and T9 after 6 h and 9 h enrichment, respectively. Samples were passed over a sterile 100-μm mesh to separate *Artemia* from culture water. *Artemia* trapped in the filter were rinsed with sterile saline water (35 ppt) and homogenized in 5 ml sterile saline water. Samples were serially diluted, then 100 μm of each dilution was spread plated on tryptone soy agar (TSA) and De Man, Rogosa, and Sharpe (MRS) agar (Liofilchem) for total viable bacteria count and LAB, respectively. Plates were incubated at 30°C for 48–72 h. After that, the amount of bacteria was counted as colony-forming unit (CFU) per nauplii for each replicate.

Experimental fish and feeding trial

Newly hatched Persian sturgeon larvae were obtained from the Dr. Shahid Beheshti Sturgeon Fish Propagation and Rearing Complex in Rasht, Guilan, Iran. They were at the stage of first feeding with a mean body weight and total length of 18 ± 1.4 mg (mean \pm SE) and 10.5 ± 0.1 mm, respectively. Water temperature, pH, and dissolved oxygen were maintained at $18 \pm 0.4^\circ\text{C}$, 7.5–8, 6–8 mg/l, respectively, throughout the trial. Water flowed continuously, and the outlet was in the center of each tank. Fish were randomly distributed into six plastic tanks containing freshwater (0.5 ppt) with 50 cm diameter and 60 l water volume at the density of 150 fish per tank (three tanks per treatment). Since *Artemia* nauplii enriched for 9 h (T9) had the

most significant CFU/g compared with the other treatments ($P < 0.05$), it was subjected to fish. Larvae in the control group were fed with nonenriched *Artemia*. Each of the two treatments (control and T9) was applied in triplicate. Larvae were fed at 30% of body weight per day at various times: 4:00, 8:00, 12:00, 16:00, 20:00, 24:00. The unfed *Artemia* nauplii were collected after the respective hours of feeding. The experiment lasted 11 days.

Growth performance and survival

To assess the growth performance, body weight and total length of 25 fish per tank were measured randomly at the end of the feeding trial. Growth performance parameters were calculated according to the following formulae: weight gain = $W_2 - W_1$; specific growth rate (SGR) = $100 [\ln W_2 - \ln W_1] / T$; condition factor (CF) = $100 - (\text{body weight; mg}) / (\text{body length; cm})^3$; feed conversion ratio (FCR) = FO/WG, where \ln = natural log, W_1 = initial weight (mg), W_2 = final weight (mg), T = time period in days, FO = feed offered (mg), WG = weight gain, BW_1 = initial biomass weight, and BW_2 = final biomass weight. Additionally, survival rate was calculated at the end of the experiment: survival = $(N_f/N_0) \times 100$, where N_0 is initial number of fish and N_f is final number of fish.

Sample collection

Fifteen fish in each treatment were taken at the end of 11 days of experiment for intestinal microbiota analysis. Fish were fasted for 24 h before the microbiological sampling. Sampled fish were killed with an overdose of 500 mg/l MS₂₂₂ (Palić et al. 2006), then the skin was washed with 0.1% benzalkonium chloride. To isolate the autochthonous intestinal microbiological communities, the entire intestinal tract was removed aseptically, washed thoroughly with sterile saline water and homogenized (Potter–Elvehjem Tissue Homogenizer, Cole-Parmer Instrument Company, IL, USA). Then, it was serially diluted to 10^{-7} with sterile salinity. Samples were spread in triplicate onto tryptone soy agar (TSA) and De Man, Rogos, and Sharpe (MRS) agar (Liofilchem), for total viable bacteria count and LAB respectively. Plates were incubated at the room temperature of 25°C for 5 days (Mahious et al. 2006) and colony-forming units (CFU/g) were calculated from statistically viable plates (i.e., plates containing 30–300 colonies) (Rawling et al. 2009).

Stress challenges

At the end of the 11 days of the feeding trial, fish were randomly sampled from each tank and transferred directly from freshwater (0.5 ppt) to saltwater for a salinity stress challenge. Persian sturgeon larvae were subjected to osmotic shocks at 15, 25, and 35 ppt, with three replicates for each treatment

(i.e., 10 fish per tank). The survival rate of Persian sturgeon larvae was calculated at 1 h post stress challenge.

The pH stress challenge was carried out at the end of the feeding trial. To achieve this, fish were removed randomly from each tank at the end of feeding trial and exposed to four different pH treatments: pH = 5; pH = 6; pH = 8; pH = 9. These pHs were achieved by adding sodium hydroxide (NaOH) to prepare alkaline water, and hydrochloric acid was added to make low pH. A portable pH meter (Metrohm, Switzerland) was used to control pH. Three replicates (i.e., 10 fish per tank) were considered for each treatment. The survival rate of Persian sturgeon larvae was calculated at 10 min post stress challenge.

To evaluate the effect of probiotics on fish resistance to air exposure, fish from each tank were sampled at the end of the feeding trial. Four treatments in three replicates (i.e., 10 fish per tank) were used. For the first treatment, all fish of each of the three replicates were held in a net for 5 s. For the second treatment, all fish of each of the three replicates were held in a net for 10 s. For the third treatment, all fish of each of the three replicates were held in a net for 15 s. All fish of each of the three replicates of the fourth treatment were held in a net for 20 s. Fish were monitored for 1 hour, and survival rate of Persian sturgeon larvae was calculated after this experiment.

Data analyses

Data are presented as means \pm standard error (SE). One Sample Kolmogorov-Smirnov and Levene's test were used to evaluate normality of data and homogeneity of variances respectively. Regression procedure was used to analyze the relation between total viable bacteria in *Artemia* nauplii and time duration. Stress resistance among different treatments were analyzed by one-way analysis of variance (ANOVA). When ANOVA identified differences among groups, Duncan's multiple range tests were performed. An independent samples test was used to determine differences between treatments in intestinal microbiota analysis and growth performance. The significance level of 0.05 was considered for data analyses.

Results

Artemia enrichment

There was a significant positive relationship between the duration of enrichment and the bacteria counts in *Artemia* nauplii on MRS agar ($r^2 = 0.67$; $P < 0.05$) and TSA agar ($r^2 = 0.91$; $P < 0.05$) (Table 1). CFU levels increased with the duration of enrichment, with the highest level observed in nauplii enriched for 9 h on both MRS agar and TSA agar.

Table 1. LAB levels (De Man, Rogosa, and Sharpe agar, MRS) and total bacterial load (tryptone soy agar, TSA) in *Artemia* nauplii enriched with probiotic *Pediococcus acidilactici* for 3 h (T3), 6 h (T6), and 9 h (T9), and in nonenriched *Artemia* nauplii (control).

	T3	T6	T9	Control
MRS (CFU/g)	6.14 ± 0.10	6.45 ± 0.05	6.96 ± 0.01	4.54 ± 0.01
TSA (CFU/g)	8.90 ± 0.2	9.23 ± 0.08	9.57 ± 0.07	8.67 ± 0.33

Values are mean ± SE.

Fish growth and survival

Growth performance and survival rate of Persian sturgeon larvae fed with nauplii enriched for 9 hours and nonenriched nauplii at the end of the feeding trial are shown in Table 2. There was no significant differences in final weight, weight gain, condition factor (CF), specific growth rate (SGR), and feed conversion ratio (FCR) between fish fed with T9 compared with the control group ($P > 0.05$). On the other hand, the survival rate of larvae fed with nauplii enriched for 9 hours increased significantly in comparison with the control group ($P < 0.05$).

Bacteriological study

LAB levels (MRS agar) in the digestive tract of fish fed with T9 were significantly elevated ($P < 0.05$) compared with the control, while no significant differences were observed in total bacterial load (TSA agar) in the digestive tract between these two groups ($P > 0.05$) (Figure 1).

Stress challenges

Probiotic enrichment (T9) significantly increased the resistance of Persian sturgeon larvae to the salinity stress challenge ($P < 0.05$) compared with the control group (Figure 2a). The survival rate of the control group after 1 h post stress gradually declined with an increase in the water salinity so that all

Table 2. Growth performance and feed utilization parameters of Persian sturgeon (*Acipenser persicus*) larvae fed diets containing *Artemia* nauplii enriched with probiotic *Pediococcus acidilactici* for 9 h (T9) and non enriched *Artemia* nauplii (control) for 11 days.

Growth performance and feed utilization parameters	T9	Control
Initial weight (g)	0.018 ± 1.4 ^a	0.018 ± 1.4 ^a
Final weight (g)	0.07 ± 0.001 ^a	0.05 ± 0.007 ^a
Weight gain (g)	0.05 ± 0.001 ^a	0.03 ± 0.007 ^a
Condition factor (CF)	1.41 ± 0.06 ^a	1.08 ± 0.01 ^a
Specific growth rate (SGR) (%/day)	4.88 ± 0.04 ^a	4.61 ± 0.16 ^a
Food conversion ratio (FCR)	4.45 ± 0.10 ^a	5.80 ± 0.03 ^a
Survival (%)	81.50 ± 0.28 ^a	41.16 ± 0.72 ^b

Values are mean ± SE. $n = 75$ per each treatment.

Data with the same superscript letters are not significantly different at the significance level of 0.05.

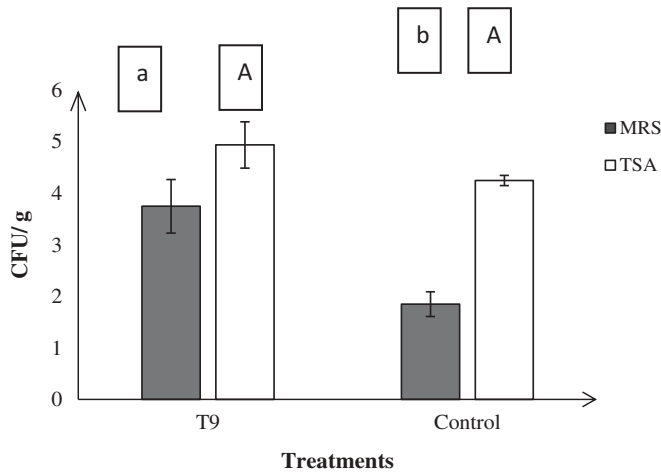


Figure 1. LAB levels (MRS agar) and total bacterial load (TSA agar) in the digestive tract of Persian sturgeon (*Acipenser persicus*) larvae fed diets containing *Artemia* nauplii enriched with probiotic *Pediococcus acidilactici* for 9 h (T9) and nonenriched *Artemia* nauplii (control) for 11 days. Values are mean \pm SE. $n = 15$ per each treatment.

larvae died at 35 ppt. The highest survival rate was observed at 15 ppt both for T9 (100%) and the control group (83.33%).

The dietary probiotic (T9) significantly elevated the resistance of Persian sturgeon larvae to the pH stress challenge compared with the control

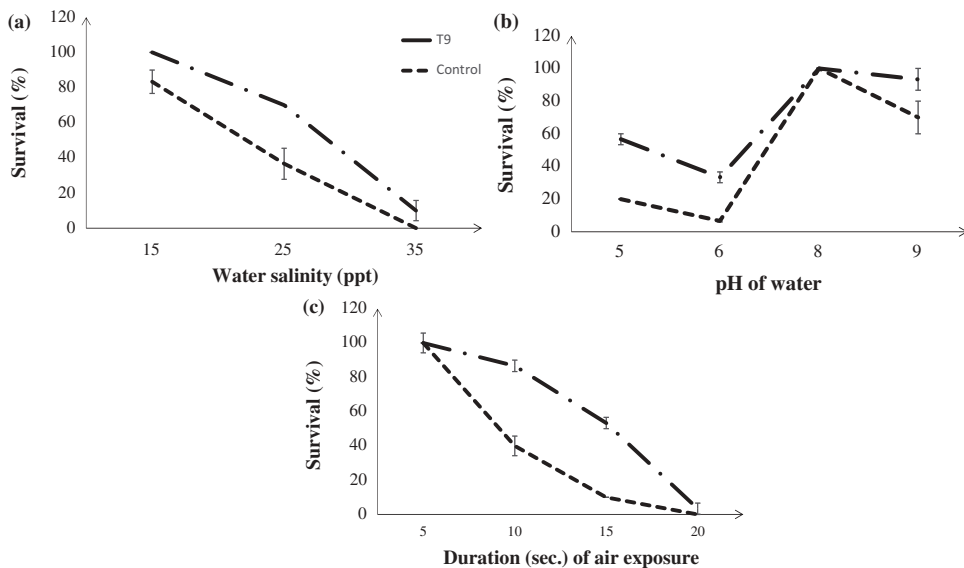


Figure 2. Survival rate of Persian sturgeon (*Acipenser persicus*) larvae initially fed diets containing *Artemia* nauplii enriched with probiotic *Pediococcus acidilactici* for 9 h (T9) and nonenriched *Artemia* nauplii (control) for 11 days, followed by salinity stress challenge (a), pH stress challenge (b), and air-exposure stress challenge (c). Values are presented as the mean \pm SD. $n = 30$ per treatment.

($P < 0.05$) (Figure 2b). The highest survival rate was observed at pH = 8 both for control group (100%) and T9 (100%).

Fish fed a diet with probiotic (T9) had the more significant resistance to air exposure than the control group ($P < 0.05$) (Figure 2c). No mortality was found in fish held in a net for 5 s either for T9 or the control, while all larvae held in a net for 20 s died for the control group, and only 10% of larvae survived for T9.

Discussion

This study was conducted to investigate the effects of *P. acidilactici* as a probiotic on the growth performance and survival rate of Persian sturgeon larvae, as well as its effects on stress resistance. The results have shown that *Artemia* nauplii enriched for 9 h had the most significant LAB level. Hence, this treatment was selected to transfer the maximum rate of *P. acidilactici* to the intestine, to enhance growth performance and survival rate of Persian sturgeon larvae. This is in agreement with our previous study on beluga sturgeon larvae (*Huso huso*), which the *Artemia* nauplii enriched for 9 h had the highest level of LAB (Zare et al. 2016). It seems that the longer enrichment time leads to the higher level of LAB.

Probiotic enrichment has been extensively used in aquaculture species (El-Haroun et al. 2006; Jafaryan et al. 2015; Lara-Flores et al. 2003; Shinde et al. 2008; Suralikar and Sahu 2001; Talpur et al. 2014; Varela et al. 2010; Ziaei-Nejad et al. 2006). There were no significant differences for CF, SGR, final weight, weight gain, and FCR between the larvae fed the diets containing *Artemia* enriched with *P. acidilactici* and the control. However, *Artemia* enrichment with *P. acidilactici* improved larvae survival compared to the control group. This result confirmed the findings of several studies that have reported the improved survival rates of fish species fed diets with probiotics (Bogut et al., 1998; Divya et al. 2014; El-Haroun et al. 2006; Faramarzi et al. 2011; Gatesoupe 1991; Seenivasan et al. 2012; Varela et al. 2010). Probiotics actively prevent the colonization of potential pathogens in the digestive tract through antibiosis, competition for nutrients and space, and alteration of the microbial metabolism (El-Haroun et al. 2006). Another explanation is possibly attributed to improvement in diet digestibility, which may cause better growth and feed efficacy associated with the enriched diets. Probiotics also affect the digestive process through enhancement of the microbial enzyme activity and the population of beneficial microorganisms and the improvement of intestinal microbial balance, which in turn improve the digestibility and absorption of food and feed utilization (Bomba et al. 2002). On the contrary, Jafaryan et al. (2015) reported poor growth rate in three spot gourami (*Trichopodus trichopterus*) fed with *Bacillus* probiotics. These contradictions are likely due to the difference in dosage, experimental fish

species, intestinal morphology, and microbiota (Hosseinifar et al., 2013). Increased survival rate in Persian sturgeon larvae fed *P. acidilactici* as a probiotic could likely be due to improved general health or immune status.

Stress challenges have been frequently used for determination of juveniles' quality in nutritional studies (Dimitroglou et al. 2010; Hoseinifar et al. 2013; Jafaryan et al. 2015; Jalili et al., 2009; Salze et al. 2008; Varela et al. 2010). The survival of both sturgeon larvae and juveniles are affected by the salinity stress, especially when they are released into the southern area of the Caspian Sea for restocking. Moreover, any alterations in water parameters beyond the optimum level have a significant influence on physiological and behavioral activities (De et al. 2014), so fish may encounter different types of stress in aquaculture systems including anoxia and fluctuation in pH. Study of the efficient osmoregulatory mechanism and water quality is, therefore, crucial to increase the survival of juveniles in rivers, estuaries, and the open sea. Our results demonstrated that Persian sturgeon larvae fed a diet containing *Artemia* enriched with *P. acidilactici* showed significantly higher resistance to salinity, pH, and air exposure stresses compared with the control group. These results agree with those previously obtained in fish species, including three spot gourami fed with *Bacillus* probiotics (Jafaryan et al. 2015), rainbow trout larvae (*Oncorhynchus mykiss*) fed with *Saccharomyces cerevisiae* (Pooramini et al. 2009), and Persian sturgeon fed with probiotic bacilli (Faramarzi, Jafaryan, Roozbehfar, Jafari, Rashidi, and Biria 2012). Enhanced host resistance to acidic pH and thermal stresses was reported by Fietto et al. (2004). Our results suggested that fish fed a diet containing *P. acidilactici* as a probiotic could increase host resistance, as probiotic bacteria could stimulate the defense system and therefore prevent the harmful effects mediated by different stress factors (Akhtar et al. 2010; Rio-Zaragoza et al. 2011).

In summary, this preliminary study revealed that administration of *Artemia* nauplii enriched for 9 h with *P. acidilactici* improves survival rate and stress resistance of Persian sturgeon. However, more work should be carried out on different aspects of probiotics administration in Persian sturgeon at an early stage of development.

Acknowledgment

The authors would like to thank the staff of the Dr. Shahid Beheshti Sturgeon Fish Propagation and Rearing Complex for providing the juvenile sturgeon.

Funding

The authors acknowledge the Pakgostar Parand Company, the Iranian branch of Lallemand Animal Nutrition, for supporting this experiment.

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