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Influences of Probiotic Bacilli via Bioencapsulated *Daphnia magna* on Resistance of Persian Sturgeon Larvae Against Challenge Tests

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Abstract: This study (4 weeks) was carried out to evaluate resistance of *Acipencer percicus* larvae against salinity, temperature, ammonia and pH stresses at the end of experimental period. *Bacillus* bacteria (*B. licheniformis, B. subtilis* and *B. circulans*) (three species in a commercial preparation, Protexin Aquatic) were bioencapsulated within *Daphnia magna* at three concentrations by holding the Daphnia in suspensions of 1×10^7, 2×10^7 and 3×10^7 bacteria per milliliter for 10 hours (T1, T2 and T3, respectively). The sturgeon larvae in experimental treatments were fed one of the three probiotic treatments at a level of 30 percent body weight 5 times a day but larvae in control treatment (C) fed by unbioencapsulated Daphnia. We prepared five stressor factors to challenge *Acipencer percicus* after 28 days feeding, trial that's included: pH (2 and 13), salinity (120ppt/l), temperature (35°C), and ammonia (2.5mg/l) challenge. Experimental larvae after feeding via bioencapsulated Daphnia by *probiotic bacilli* gave a relative percentage survival rate in comparison with control treatment ($P < 0.05$). The analyses were based on challenge test data (as seconds) from four treatments. In conclusion, bioencapsulated Daphnia with probiotic bacilli could enhances the resistance against stresses in *Acipencer percicus*.

Key word: *Acipencer percicus* · Persian Sturgeon · *Daphnia magna* · Ammonia · Challenge · Bioencapsulation

INTRODUCTION

Live food (as *Daphnia magna*) have been used as vectors for delivering compounds of diverse nutritional and/or therapeutic value to larval stages of aquatic animals [1], a process known as bioencapsulation. The *Daphnia magna* is 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. Intensive rearing of marine fish larvae suffers from heavy mortalities, which may be attributed to bacteria introduced in the rearing system with live food [2]. 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 [3], growth [4], stress resistance [5], immunosystem enhancement [6], and finally general welfare [7].

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 biotics 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 [8]. The use of natural immunostimulants is promising in aquaculture because they are safe for the environment and human health, biocompatible and biodegradable [9]. Many studies have looked into the modulation of the immune system in fish as a means to prevent disease outbreaks [10].

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Finding a definition for stress is a difficult task. Levin [11] reported that it is often quoted as being uncertain whether anyone attempting to define stress "either has an enormous ego, is immeasurably stupid, or is totally mad." The general definition by Selye [12], stating that stress is the response of an organism to any demand placed on it such that it causes an extension of a physical state beyond the normal resting state, may be the most useful when considering fish in culture systems. Challenge tests are proposed as meaningful tools for assessing fish quality in the aquaculture industry, environmental resources management and in research [13]. The concept is based on the presumptions that stress loading above the acclimation capacity of an organism will weaken it and reduce performance in growth, survival and reproduction, and that the reduction in performance can be quantified by assessing tolerance to reference stressors [14]. Performance tests which are described included; thermal, hypoxia, chemical and salinity tolerance tests, swimming performance, resistance to disease, crowding and handling tests. Literature on the use of stress challenge tests for determining finfish quality is scarce. Salinity challenges have been used to determine the hypoosmoregulatory capability (smoltification) of juvenile anadromous salmonids [15].

The aim of this study was to determine the effects of probiotic bacilli against stressor factors such as pH, salinity, temperature and ammonia in Acipenser persicus larvae after 28 days feeding by bioencapsulated Daphnia.

MATERIALS AND METHODS

Experiment Design: The probiotic bacillus was prepared from the commercial product Protexin aquatic (Iran-Nikotak), which is a blend of three *Bacillus* species. The blend of probiotic bacilli (*B. licheniformis, B. subtilis* and *B. circulans*) from suspension of spores with special media was provided. Three concentrations of bacterial suspensions, 1×10^7, 2×10^7 and 3×10^7 CFU mL^{-1} were provided by Protexin Co and the colony forming units (CFU) of probiotic bacilli were tested by microbial culture in styptic soy agar (TSA).

Daphnia magna were obtained from intensive production ground ponds of the center of sturgeon culture of Marjani (Iran). The *Daphnia magna* at a density of 5 g live *Daphnia* $litter^{-1}$ was held in a broth suspension with *Bacillus circulans*, *Bacillus subtilis* and *Bacillus licheniformis* at densities of 1×10^7, 2×10^7 and 3×10^7 bacteria per milliliter for 10 hours.

This experiment was conducted in a completely randomized design with four treatments (three probiotic levels and a control), and three replicates per treatment for a total of twelve fiberglass tanks (each with a capacity of 40 liters). Larvae of Persian sturgeon (initial weight: 74.9±0.89 mg) were obtained from the center of sturgeon culture of Marjani (Iran). The density of fish larvae in per tank were 71 fish. Persian sturgeon larvae in control and experimental treatments were fed 30 percent of their body weight for 5 times a day (2.00, 7.00, 12.00, 17.00 and 22.00??). The control treatment was fed unbioencapsulated *D. magna*. Water quality parameters of input water to rearing system were monitored each week throughout the experiment. The water temperature was 19.46±1.23°C, pH was 7.85±0.26 and water oxygen level was maintained above 7.65±0.55 mg L^{-1} during the experiment with an electrical air pump (by a single filtration unit).

Range-Finding Tests: To determine the range of concentrations to be used for 10 minute (LC50 testing), larvae which had been conditioned for packing were exposed to salinities of 0 (control), 30, 60, 90, 120 and 150 ppt for 10 minute. For temperature range, larvae were exposed to 20, 25, 30, 35 and 40°C. To determine the range of pH, five concentrations of 11, 11.5, 12, 12.5 and 13 ppm (for alkalinity challenge) and five concentrations of 1, 1.5, 2, 2.5 and 3 ppm (for acidity challenge) were used and for assign range of ammonia, *,Acipenser persicus* larvae were exposed to concentrations of 1, 1.5, 2, 2.5 and 3 mg/l. three replicates of each concentration were used. Mortality at 10 minute was noted for calculation of LC50.

PH Challenge: For pH challenge after feeding trial 60 fish (5 fish than each replicate) were exposed to natural acid (average pH 2) and non-acid source water (average pH 13) and water added sulfuric acid (H_2SO_4) to increase toxicity or limestone or sodium-silicate to reduce toxicity. At the period of challenge pH was checked by pH meter. After exposure, larvae mortality was observed.

Salinity Challenge: A 120 ppt saltwater (SW) static aquarium was produced by adding salt to freshwater from the fish's environment. Test fish were quickly captured and placed in the SW aquarium. Three groups of experimental feeding fish and one group of control fish were placed into separate tank. At the end, dead time of fish was recorded as second.

Thermal Challenge: Five Acipenser persicus larvae were removed from each tank. The larvae were exposed to heat shock (35°C). Tank water temperature was monitored minutely from beginning to end of the experiment. After heat stress, larval resistance and mortality against stressor was recorded. Death was defined as the point at which fish lost balance.

Ammonia Challenge: To examine the effect of high ammonia exposure, five *Acipenser persicus* larvae from each tank were challenged with elevated water ammonia. Acipenser persicus larvae were exposed to 2.5mg/l for challenge (pH 7.5) during exposure. At the end of the exposure period, time of resistance against stressor (per second) was recorded.

One-way ANOVA and Duncan's multiple range tests were used to analyze the significance of the difference among the means of treatments by using the SPSS program.

RESULTS AND DISCUSSION

The stress parameter levels are indicated in Figure 1-5. Our results revealed that, probiotic bacilli has been shown to enhance the non specific cellular immunity which was significantly increased in experimental treatments at 4th week post treatment in comparison with the control group (P<0.05). It was observed that the mortalities among the challenged fish are dose related. Generally, mortality rates post challenge tests, were significantly lesser in experimental treatments in comparison with control (P<0.05). In the first experiment (pH challenge), the mortalities of larvae fed with probiotic bacilli were significantly lower than the mortality of control group for both of alkalinity and acidity challenge (P<0.05). Moreover, in this experiment, Acipencer percicus larvae showed higher resistance for exposure to the acidity condition compared to alkalinity environment. In the second experiment (thermal challenge), there was no significant difference in resistance between experimental treatment and the control in thermal challenge (P>0.05). In the third experiment (salinity challenge), the mortality of larvae fed with probiotic was also significantly lower than the control group. In the fourth experiment (Ammonia challenge), Fishes used for ammonia exposure showed significant differences in mortality and resistance (P<0.05). The larvae witch were fed with enriched daphnia had higher ability to resistance against stressor comparison larvae were fed not enriched daphnia (control).

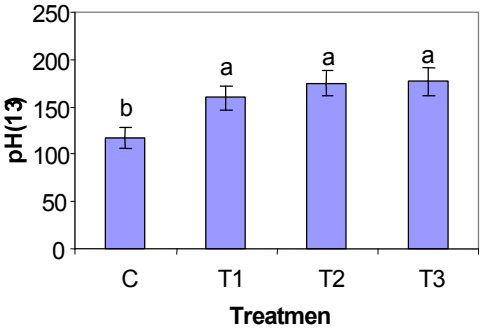

Fig 1: Evaluation of resistance in larvae against pH (13) challenge based on second (p<0.05)

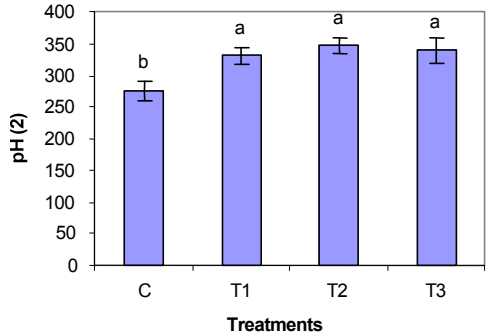

Fig. 2: Evaluation of resistance in larvae against pH (2) challenge based on second (p<0.05)

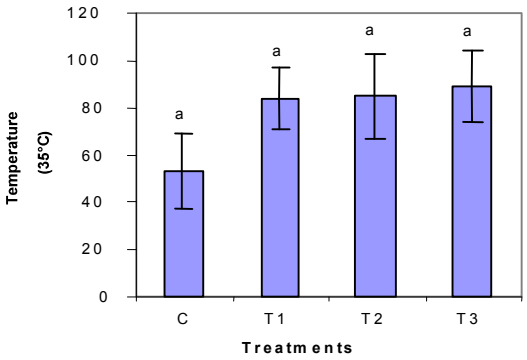

Fig 3: Evaluation of resistance in larvae against temperature (35°C) challenge based on second (p>0.05)

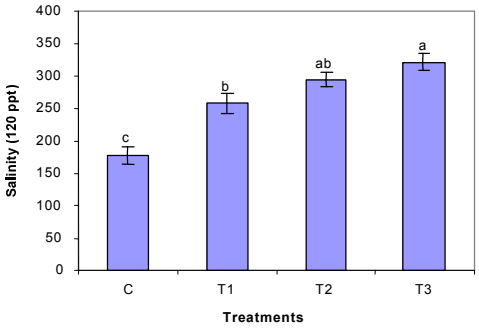

Fig. 4: Evaluation of resistance in larvae against salinity (120 ppt) challenge based on second (p<0.05)

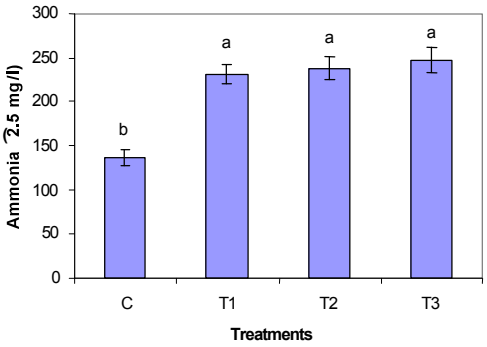

Fig. 5: Evaluation of resistance in larvae against ammonia (25 mg/l) challenge based on second ($p<0.05$)

Generally our result showed that probiotic bacilli had significantly positive effect on resistance of Persian sturgeon larvae in experimental treatments in comparison with control treatment ($P<0.05$).

The effects of probiotics have been widely studied in cultured aquatic species, particularly the enhancement of the non-specific immune system [16] which is favorable to pathogen control [17]. Probiotics are particularly interesting, because they provide *β- glucan* and nucleotides that stimulate the immune system of fish [18]. There is an evidence that the administration of *glucan* extracted from the cell wall of *Saccharomyces cerevisi*ae induces increased resistance to infection by *Vibrio anguillarum*, *V. salmonicida* and *Yersinia ruckeri* in Atlantic salmon (Salmo salar) [19] Another study also showed an increase of the survival and digestive enzyme activity in *Dicentrarchus labrax* larvae fed diet containing *Debaryomyces hansenii* [20]. This investigation demonstrated that *β- glucan* and nucleotides might be useful to increase disease resistance of fish. When these immunostimulants were added to the feed, one possible explanation for the results of the first experiment is that the mechanism of action of these compounds may be through the stimulation of the nonspecific immune system, which has been observed in other investigations [21].

Several studies have also observed increased disease resistance in fish fed diets supplemented with probiotic bacilli. Kumari and Sahoo [22] reported the capacity of probiotic or structural polysaccharides to improve disease resistance in fish showed by their capacity to reduce mortalities associated with infection by pathogens such as *Aeromonas*.

It is important to develop educational and demonstrative programs for commercial fish farmers to promote and demonstrate the efficacy of preventive medicine that include also the use of vaccine and immunostimulants, instead of the mass application of drugs and antibiotics.

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