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Research Article

The effects of four types of specific probiotic on growth performance, liver enzymes and immune indices of juvenile Persian sturgeon (*Acipenser persicus*)

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Abstract

In the present study, the effects of specific and native probiotics were examined on growth factors, liver enzyme indices, and immunity of Persian sturgeon (*Acipenser persicus*) in the juvenile stage. Three hundred juvenile Persian sturgeon with a mean weight of 92.5±0.5 g fed with 4 different diets (3 replicates for each) were in 500-l fiberglass tanks (25 fish per tank) for 8 weeks. Treatments included: 0 (control), 1.5×10^7 (treatment 1), 3×10^7 (treatment 2) and 4.5×10^7 (treatment 3) CFU kg^{-1} specific probiotic per kg of feed. At the end of the experiment, the results indicated that there were remarkable differences between treatments, in terms of condition factor, food conversion ratio, specific growth rate, and weight gain percentage compared to the control group ($p<0.05$). In addition, no mortality was observed in probiotic-treated groups. There was no significant difference between probiotic and control groups in liver enzymes included ALT, AST, ALP, and LDH ($p>0.05$). IgM and lysozyme were significantly higher in probiotic-fed groups compared to the control group ($p<0.05$). Overall, the results indicated that growth factors, biochemical and immunity indicators of fish fed with diet containing specific probiotic and mainly treatment 2 (3×10^7 CFU kg^{-1}) were in the acceptable condition in comparison to the control group.

Keywords Growth factors, Probiotic, *Acipenser persicus*, Liver enzymes, Immune indices

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Introduction

The value of probiotics in field of food and medical health created a new category. In 2001, the FAO/WHO announced a new definition of a probiotic "live microorganisms which when administered in adequate amounts confer a health benefit on the host" since then, this particular meaning of probiotic has become the most accepted version worldwide. Evidence of a health benefit is required for a probiotic, at either a strain-specific or group level, depending on the nature of the benefit. Probiotics can have different means of administration, target host species (humans and animals), target populations, target sites (gut and beyond), efficacy end points and regulatory categories. All probiotics must be safe for their intended use. Dead microbes, microbial products, microbial components do not come under the probiotic classification according to Hill *et al.* (2014). These properties are not limited to human probiotics, but can also be used for aquaculture.

Due to the disappearance of the natural habitat of some fish species that cause their extinction sturgeons are no exception, one of these economically valuable fish is Persian sturgeon (*Acipenser persicus*) belongs to Acipenseridae family as a native species of the southern Caspian Sea (Darafsh *et al*., 2020). These fish natural stocks have declined 50% over the past 47 years, its catch has dropped from 440 tons in 1960 to 137 tons in 2007 (Afraei Bandei *et al.*, 2014) and listed on the IUCN red list since 2010. In recent years, Persian sturgeon has been one of the most important species in the centers of reproduction and regeneration aquaculture farms in Iran. Enormous amounts of adults are artificially reproduced each year and offspring are released to the rivers to rebuild genetic resources (Chakmehdouz *et al.*, 2014).

More than 50% of the cost of rearing is related to nutrition, and most research in this area relates to nutrition strategies and nutrient optimization for important commercial fish species. However, several factors are involved in the process of fish rearing in aquaculture, environmental factors and various diseases can have a deterrent effects on the breeding process (Lara-Flores *et al*., 2003; Panigrahi *et al*., 2004; El-Haroun *et al*., 2006; Yanbo and Zirong, 2006; Ferguson *et al*., 2010; Faramarzi *et al*., 2011; Pour ali *et al*., 2011; Soltani *et al*., 2019). In modern aquaculture, it has been proven that in addition to proper concentrated food, using an appropriate probiotic as a dietary supplement leads to maintain intestinal microbiota balance and compete with pathogens by enhancing host immune factors. Probiotic can directly and indirectly affect aquatic health and growth. Probiotic includes bacteria, yeasts, bacteriophages, and single-celled algae, the most common of which are bacterial probiotic. Different species of lactic acid producing bacteria have a special place in this regard, these bacteria are gram-positive, non-motile, spore-free and catalase-negative, with lactic acid production as the final and unique by-product of the fermentation metabolism

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(Ringo and Gatesoupe, 1998). Numerous and considerable studies in aquaculture used the probiotic potential of lactic acid bacteria to achieve a variety of goals including reducing infectious diseases, reducing antibiotic use, improving gonadal index and fertilization in adults, improving microbial balance of fish digestive tract, proliferation control and activity of pathogenic bacteria, stimulate the immune system, reduce stress and susceptibility to diseases, improve growth and feed conversion ratio and reduce mortality (Balcazar *et al*., 2007; Soltani *et al*., 2008; Nayak, 2010; Mahmoudi *et al*., 2011). This has been proven through numerous researches**.** In microbial studies, native species isolated from aquatic digestive tract, have major impact on aquaculture purposes. Alizadeh *et al.* (2017) showed that *Enterococcus faecalis* as a native probiotic isolated from *A. persicus* had significant improvement in blood and serum factors. Also another study from Shenavar Masouleh *et al* (2016) showed positive results of using *Lactoccocus lactis* on the status of the intestinal bacterial flora of *A. persicus*. It was noted in the above study that in the third treatment with 10^8 CFU/g probiotic intake, leaded to significantly higher survival rate than the control and other treatments**.** In another study, Moslehi *et al.* (2015) investigated the effects of *Pediococcus pentosaceus* as a probiotic on Siberian sturgeon (*Acipenser baerii)*. The results of this study showed that *P. pentosaceus* could significantly improve growth factors such as FCR, BWI, PER and SGR, and also the immune system. Soltani *et al.* (2013) isolated specific probiotics from Persian sturgeon intestine including *P. pentosaceus, L. lactis, E. faecalis* and *Weissella cibaria* and they identified these species from 16sRNA and registered their genetics in NCBI. Few studies have been conducted on the specific use of probiotic on sturgeon, therefore, the aim of this study was to investigate the use of specific probiotic in Persian sturgeon in juvenile stage to evaluate the effects of probiotic on growth and immunity.

Materials and methods

Probiotic and preparation of experimental diet

The dried probiotic powder used in this study included blend of *Pediococcus pentosaceus, Weissella cibaria, Lactoccocus lactis* and *Enterococcus faecalis* with the same dosage of each (10^8 CFU kg^{-1}) was produced by International Sturgeon Research Institute (Rasht, Iran).

Experimental design

Three hundred Persian sturgeon with mean weight of 92.5 ± 0.5 g were obtained from the International Sturgeon Research Institute and randomLy distributed in 12 tanks of 500 liter fiberglass with total volume of 350 liters (25 fish per tank). Treatments included 0 (control), 1.5×10^7 (treatment 1), 3×10^7 (treatment 2) and 4.5×10^7 (treatment 3) CFU kg^{-1} specific probiotic which considered to fed the fish for 8 weeks.

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Preparation of experimental diets

According to the nutritional requirements of Persian sturgeon, concentrated feed based on basal diet (produced by Coppens Co, Netherland) was used in this experiment, containing the main compositions such as: 45% crude protein, 18% crude fat, 1.8% cellulose, 9.5% ash, 1.49% phosphorus, 1.85% calcium , 0.55% sodium, vitamin A 12000 (IE / kg), vitamin E 240 (mg / kg), copper 1.5 (mg / kg), manganese 12 (mg / kg), zinc 75 (mg / kg) and iodine (1.8 mg / kg). To supplement the feed, the bacterial strains (as described above) were weighed under a laminar hood in sterile plastic containers, and 50 mL of sterile physiological serum was added, then sprayed on special dishes and then fed. The containers were kept in the refrigerator. After transferring the fish to the farming tanks and adaptation period, they were fed by different diets for 8 weeks. The feeding amount was determined based on the fish's appetite. After biometry, the feeding rate was calculated according to 2% of body weight, and then for the next six weeks of feeding was determined at 2.5% of body weight. Fish were fed 3 times per day at 8:00, 16:00, and 24:00.

Water condition

Inlet water flows continuously through the pips above the tanks, at a flow rate of 4 l/min, the water outlet was in the center of the tank. Environmental factors such as water temperature (^{o}C), dissolved oxygen (mg/L) and pH were measured during the culture period (Table 1).

Table 1: Physical and chemical factors of water measured during the experimental period (mean±SD)

pH	Dissolved oxygen (mg/L)	Water temperature (^{o}C)
8.1±0.23	7.14 ± 0.62	19.27±0.88

Sample collection

At the end of the experiment, to reduce stress, feeding was discontinued 24 hours before biometric.

Growth performance

Initial biometric were performed before the starting rearing period, fish weight and length were measured by digital scale with 0.01 g accuracy and biometric board with 0.1 cm accuracy, respectively. At the end of period, all fish were removed from each tank to evaluate the growth performance indices including food conversion ratio (FCR), specific growth rate (SGR), condition factor (CF), body weight increased (BWI) and which were determined by following formulas:

FCR= food consumed (g)/ weight gain (g) (Hevroy *et al*, 2005)

SGR= (ln final weight - ln initial weight)/times period in days ×100 (Hoffman *et al*, 1997)

CF= (fish weight (g)/fish length (cm^3) ×100 (Ojolick *et al*, 1995)

BWI (%)=(final weight-initial weight)/initial weight ×100 (Hung *et al*, 1989)

SR (%)=$n_{final\ fish}$ / $n_{initial\ fish}$ × 100 (Mazurkiewicz *et al*, 2008)

Blood sample collection

At the end of the feeding period, to assess the effects of probiotic on liver

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enzymes and immune parameters of Persian sturgeon, 9 fish from each group were anaesthetized with clove solution and then were sampled randomLy (36 samples in total). Following the fasting of fish for 24 h blood was drawn from the caudal vein at the end of the anal fin using a 2 mL syringe. 1.5 mL of blood were transferred to non-heparinized tubes in order to measure liver enzymes and immune parameters (Bazari Moghaddam *et al.,* 2017). The samples were centrifuged using centrifugation at 2500 rpm for 10 minutes and then upper layer serum were transferred to a new tube and stored at -80°C. Preparation and measurement of all serological parameters were carried out at the International sturgeon research Institute and Viromed laboratory.

Liver enzymes assay

Enzyme activity such as alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), lactate dehydrogenase (LDH) were determined using diagnostic kits (www.parsazmun.com) by BT-1500 auto analyzer (Instruments Biotecnica Italy) (Shahsavani *et al.,* 2008).

Immunological assay

Lysozyme activity

Lysozyme level was accomplished according to the Sahoo *et al*. (2008) with insignificant modifications. Using ELISA Reader (Awarness, USA, Model 2100-Staphax) by turbidimetric method, gradual analysis of gram positive *Micrococcus lysodeikticus* bacteria (Sigma, USA) was obtained. Thus, plasma (50 µL) was added to 2 mL of a suspension of *M. lysodeikticus* (0.2 mg/mL^{-1}) in a 0.05 M sodium phosphate buffer (pH 6.2). The reaction was carried out at 25ºC and absorbance was measured spectrophotometrically at 570 nm after 0.5 min and 4.5 min. PBS was used as a blank. A unit of lysozyme activity was defined as the sample amount causing a decrease in absorbance of 0.001 min^{-1}. Lysozyme of sample calibrated using a standard curve determined with hen's egg white lysozyme (Sigma) in PBS (Bazari Moghaddam *et al.,* 2017).

Immunoglobulin M (IgM)

The IgM content was analyzed using the immunoturbidimetry kit according to the method described by Khoshbavar-Rostami *et al.* (2006). Briefly, IgM is complex with polyclonal antibodies in the tampon solutions, causing the solution to become opaque. The turbidity intensity was directly related to IgM and was observed by a spectrophotometer (Model 2100 – VIS, Unico USA) at 320 nm with Planck (distilled water).

Statistical analysis

Statistical analyses were performed using SPSS (version 20) software. In order to investigate the normal distribution of data for the formation of treatments from the experiment, we used Kolmogorov-Smirnov test. Data are presented as mean±SD. Statistical comparison of treatments was performed

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by One-way analysis of variance (ANOVA) at 95% level. The significant means were compared by Duncan test. All tests used a significance level of p=0.05.

Results

At the beginning of the experiment, there was no significant difference between the mean initial weight and length of the fish (p>0.05). The mean final weight and length, although increased, however no significant differences were observed (p>0.05).The effects of different levels of dietary probiotic blend showed significant differences (p<0.05) on the growth performance including FCR, SGR, CF and BWI in fish fed probiotic compared with control group (Table 2). Liver indices such as ALT, AST, ALP and LDH in fish exhibited normal behavior under both the low and high densities of probiotic compared to control and there were no significant differences (p>0.05) in these parameters among treatments (Table 3). In this study, the result of immune parameters (Table 3) showed that lysozyme and IgM levels in different treatments supplemented with probiotic were significantly different (p<0.05) compared to the control group.

Table 2: Growth performance of juvenile Persian sturgeon fed with different levels of probiotic for 8 weeks (mean±SD).

Growth performance Parameters	Levels of probiotic (CFU kg^{-1})			
	Control (0)	1.5	3	4.5
Initial mean weight (g)	92.59±0.87	91.37±0.86	93.24±0.34	93.40±0.27
Initial mean length (cm)	29.97±0.08	29.74±0.02	30.06±0.04	30.14±0.16
Final mean weight (g)	222.72±6.62	229.43±10.45	237.15±3.79	238.02±3.60
Final mean length (cm)	38.38±0.3	39.06±0.52	39.71±0.31	39.76±0.20
FCR	1.23±0.01^{a}	1.19±0.03^{ab}	1.17±0.04^{b}	1.16±0.04^{b}
SGR (%/day)	1.56±0.04^{b}	1.64±0.08^{a}	1.66±0.03^{a}	1.67±0.07^{a}
CF (%)	0.45±0.003^{c}	0.48±0.002^{b}	0.48±0.004^{b}	0.51±0.004^{a}
BWI (%)	140.48±5.69^{b}	161.91±2.81^{a}	154.33±3.79^{ab}	146.33±5.30^{b}

Data are expressed as mean±SD. Different letters stand for significant differences at p<0.05.

Table 3: Biochemical and immunological parameters of juvenile Persian sturgeon fed with diet containing different levels of probiotic for 8 weeks (mean±SD).

Liver enzymes & Immune indices	Levels of probiotic (CFU Kg^{-1})			
	Control (0)	1.5	3	4.5
ALT (u/L)	41.17±2.74	40.67±4.36	39.83±3.35	41.33±2.22
AST (u/L)	480.33±42.81	435.67±43.02	427.17±53.49	479.30±41.59
ALP (u/L)	588.67±22.86	569.17±31.89	571.23±31.71	591.12±45.79
LDH (u/L)	931.83±33.78	935.50±35.80	929.62±108.09	932.20±34.83
Lysozyme activity (µg/mL^{-1})	22.67±1.17^{b}	33.83±1.27^{a}	33±1^{a}	29.83±1.95^{a}
IgM (mg/dl)	26.67±0.8^{c}	32±1.37^{b}	32.33±1.40^{b}	42.33±1.96^{a}

Data are expressed as mean±SD. Different letters stand for significant differences at p<0.05.

Discussion

Previous studies and this study have shown that bacteria isolated from the intestine of a particular species of sturgeon and its use as a probiotic supplement in feeding of the same species have more beneficial effects on growth and immunity. Studies have shown that probiotics are detoxified food ingredients and alteration of the

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nature of non-digestible compounds by hydrolytic enzymes includes amylase, protease and vitamin production such as biotin and vitamin B12 (El-Haroun *et al.,* 2006) and also it can increase their competitiveness against pathogens and play a protective role. Manipulation of the intestinal microflora by the use of probiotics can be a valuable mechanism to increase growth and survival rate of fish. In a study by Aly *et al.* (2008), it was found that the addition of probiotic *Lactobacillus acidophilus* to the diet of tilapia (*Oreochromis niloticus*) significantly increased the growth. Similar results were observed in the study of the effect of *W. cibaria* on Siberian sturgeon (*A. baerii*) after 60 days of feeding with probiotic and growth indices including FCR, SGR, PER and BWI with 10^8 CFU/g showed significant differences compared to control group (Hashemimofrad *et al.,* 2017). The present study presented the same results that showed significant differences in FCR, SGR, CF and BWI in almost all probiotic treatments, especially treatment 2 with 3×10^7 CFU kg^{-1} of probiotic. In this study, the results showed that at different levels of probiotic, feeding of native lactic acid bacteria can increase efficiency, which is more stable than the control group due to 100% survival. A similar results have been reported by Panahi-Sahebi *et al.* (2019) which was showed that *Cyprinus carp* without a probiotic diet had a lower survival rate. In contrary of our study, Ferguson *et al.* (2010) examined the effect of *Pediococcus acidilactici* on growth indices in red tilapia (*O. niloticus*)and found that there were no significant differences in FCR, SGR and PER in control group compare to the probiotic fed groups. However, differences in this study and the present study may be due to factors such as differences in probiotic type, host species, probiotic content, genetic structure, and duration of testing (Nikoskelainen *et al*., 2003; Salinas *et al*., 2005; Son *et al*., 2009). Improve FCR in this study and other studies (Lara-Flores *et al.,* 2003; Yanbo and Zirong, 2006) shows that if we use probiotic supplements, we can reduce the amount of food needed to grow fish and reduce production costs.

ALT, AST, ALP and LDH enzymes are crucial tools in determining the health status of fish. Liver cells are rich in enzymes and are used to evaluate liver tissue damage, changes in serum enzymes are related to a variety of diseases, parasitic infections and poisonings. In the present study, ALT and AST values in treatment 2 were alleviated compared to control group but not significantly. Results showed that ALP values in treatments 1 and 2 were lower than control, but treatment 3 had an increasing trend. Also LDH levels showed no differences between treatments and control at the end of experiment. However, practically treatment (3×10^7 CFU kg^{-1}) was reduced compared to all treatments. Contrary to the present study, the study of Alizadeh *et al.* (2017) showed a significant difference on liver enzymes indices

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including AST and ALT in Persian sturgeon larvae. In the present study, the use of probiotic did not significantly increase liver enzymes compared to the control, which may indicate an ineffective of probiotic in Persian sturgeon.

Lysozyme activity is considered as an immunological factor (Kim and Austin, 2006). This enzyme increases combined with injections of microbial preparations in response to bacterial infections and a probiotic-rich diet in fish serum (Tokmechi, 2007). Lysozyme, being an enzyme with antibacterial activity, can split peptidoglycan in bacterial cell walls especially the gram positive species and can cause lysis of the cells, The results in this study showed that lysozyme activity in probiotic treatments including *P. pentosaceus, E. faecalis, W. cibaria* and *L. lactis* were more exceeded than the control treatment and there was a significant difference at the end of the feeding period. Similar results were observed in other studies (Son *et al*., 2009; Geng *et al*., 2011). Also this result is concert with increase in lysozyme activity due to administration with different amounts of probiotics supplementation diet in *Paralichthys olivaceus* (Harikrisnan *et al.,* 2011). According to Panigrahi *et al.* (2004), they were obtained almost the same result from the effects of induced potential probiotic bacteria *Lactobacillus rhamnosus* on *Oreochromis mykiss* immune response. In that study the LAB fed groups showed elevated level of lysozyme activity, but the group receiving the higher density showed to have significantly higher lysozyme activity compared to that of the control indicating activation of the immune system (Panigrahi *et al.,* 2004), but in contrary to our study the group which received lower density of probiotic (1.5×10^7 CFU kg^{-1}) compared to treatments 2 (3×10^7 CFU Kg^{-1}) and 3 (4.5×10^7 CFU kg^{-1}) had higher amounts of lysozyme. Immunoglobulins are well recognized to provide disease protection in animals and human beings and some studies have also shown the effect of lactic acid bacteria on enhancing factors like IgM (Panigrahi *et al.,* 2004). Immunoglobulin analysis of fish is important to improve immunity against infectious diseases. In sturgeon culture under dense conditions, and infectious diseases such as *Aeromonas* have been reported (Khoshbavar-Rostami, 2006). The results of this study showed that probiotics significantly increased the IgM level, and this increase is evident in all 3 probiotic-fed treatments. Our results were in agreement with Alizadeh *et al*. (2017), which they examined the effect of *E. faecalis* on nonspecific and specific immune system to determine the effect of probiotic diet on Persian sturgeon. They have noted that all treatments had a significant stimulation (Lysozyme and IgM) compared to control group (Alizadeh *et al.,* 2017).

According to the results, it can be concluded that the growth indices in this study were completely affected by diets containing different concentrations of specific probiotic and positive changes were observed. The probiotic intake was better than the control group in terms of

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maximum changes in growth indices such as FCR, SGR, BWI and CF in treatment 2 (3×10^7 CFU kg^{-1}). Moreover, analyzing the results of liver enzymes and serum immunological indices showed great performance of probiotics especially treatment 2 (3×10^7 CFU kg^{-1}) on these factors. Overall, the results of the present study indicate that the use of specific bacteria isolated from Persian sturgeon including *P. pentosaceus, E. faecalis, W, cibaria and L. lactis* in the same mixture (10^8 CFU kg^{-1}) promoted growth performance, liver enzymes and immunological indices in juvenile Persian sturgeon and these probiotics can be used in combination as a balancing agent for intestinal microbial flora to enhance their immunity of Persian sturgeon.

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