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Research Article

Effects of adding two native bacterial strains (*Lactococcus lactis* and *Weissella confusa*) on growth performance, immune indices, and intestinal flora of juvenile great sturgeon (*Huso huso*)

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Abstract

This study was carried out to determine effects of diets supplemented with two bacterial strains *(Lactococcus lactis* and *Weissella confusa)* on the growth performance, immune indices and intestinal microflora of great sturgeon juveniles. At the beginning of the feeding trial, the mean weight (±SD) of the fish was 79.44±3.18 g. At random, 15 fish were stocked per each fiberglass tank (1m×1m×0.5m) containing 300 L freshwater. The diets were prepared through spraying 50 ml bacterial suspensions containing 150, 300, and 450 mg of the bacterial strains per kg of pelleted diets to make certain concentrations 1.5×10^9 cfu/g (T_1), 3×10^9 cfu/g (T_2), 4.5×10^9 cfu/g (T_3). The blood neutrophils in the T_1 and T_2 significantly increased as compared to the control group and T_3. Lymphocytes in the control and T_3 were significantly more than T_1 and T_2. However, eosinophils showed no change between the fish fed with the supplemented diets and control group. Monocytes in T_3 considerably decreased when compared to T_1, T_2, and control. IgM and C_3 in the experimental treatments were significantly higher than the control. Lysozyme, C_4, and ACH50 in T_1 an T_2 were significantly higher than T_3 and control. Colony count of lactic acid bacteria in the intestine of fish in T_1 and T_2 was significantly higher than the control and T_3 groups. Colony count of the aerobic and facultative anaerobic bacteria in the intestine of fish in the medium of TSA in control was significantly more than T_1 and T_2. Since the TSA medium is a kind of non-selective environment and provides sufficient nutrients for a wide range of microorganisms, the medium indicated that intestinal microflora condition was worse in the control fish. The growth performance indices (weight gain, biomass increase, specific growth rate, daily weight gain, and condition factor) demonstrated no significant difference between treatments and control. There was no significant difference in term of FCR between control, T_1, and T_3. Overall, it can be stated that the two bacterial strains could induce favorable influence on intestinal microflora, immune indices, biochemical parameters, and growth performance at two levels of 150 mg (T_1) and 300 mg (T_2) especially in the T_2.

Keywords: *Huso huso*, Probiotic, Growth performance, Intestinal microflora, Immune indices

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Introduction

Great sturgeon (*Huso huso*) possess special features including high growth rate, facile adaptability to controlled environment conditions, and high value of meat and caviar. This species may be infected with several harmful bacteria at unfavorable conditions such as low water quality and high density. Therefore, special attention has been paid to probiotics, prebiotics, and synbiotics to improve the conditions of aquaculture farms (Adel *et al*., 2016).

Probiotics are known as unabsorbable dietary supplements that modulate mucosal, systemic immunity and improve the intestinal microflora balance through preventing colonization of undesirable bacteria. Probiotics have a positive effect by increasing the ratio of food intake (Zare *et al*., 2017). Probiotics can be used individually or multilaterally via adding into water and diet. In general, aquatic organism's immune systems are affected by periodic and unforeseen changes in their environment. Undesirable environmental conditions can cause stress in fish and adverse effects on biochemical parameters, innate and adaptive immune responses. Fish are more dependent on nonspecific defense mechanisms than mammals (Yanbo and Zirong, 2006).

Probiotics as a useful bacterial population can improve fish immunity under adverse environmental conditions by modify the colonization of probiotic bacterial strains as well as production of antibodies, acid phosphatases, lysozyme, and gastrointestinal antimicrobial peptides (Abareethan, and Amsath., 2015). Also, probiotics can improve resistance to disease by immunomodulation (Safari *et al*., 2016).

One of the bacterial strains used in the present study is *Lactococcus lactis* (a gram-positive bacterium). Soltani *et al*. (2016) reported that *L. lactis* can act as a positive probiotic in Persian sturgeon (*Acipenser persicus*) by improving growth performance, nutrition coefficients and fish health. Nguyen *et al*. (2018) investigated the effect of *L. lactis* as probiotic on growth and low molecular weight metabolites of olive flounder *(Paralichythys olivaceus)* and reported its effectiveness. Sharma *et al*. (2018) reported the probiotic properties of *Weissella confusa* (a gram-positive bacteria) to improve growth and survival and resistance to acidic, lysosomal and heat environment. The authors reported that *W. confusa* has the ability to bind to the digestive system, as well as its anti-oxidant and beta-galactosidase activities, cholesterol transfer and thus promote health. Rangpip *et al*. (2008) reported that sea bass fed with the bacteria *W. confusa* had more significant growth than other probiotics. Also, the strain *W. confusa* was used as a probiotic in the diet of Siberian sturgeon diet (Hashemifard *et al*., 2017). Shenavar Masouleh *et al*. (2017) reported that lactic acid bacteria (LAB), *W. confuse*, and *L. lactis* not only can resist against acid and bile, but also they can produce extracellular enzymes, including amylase, lipase, proteinase, and cellulose. They can be used as probiotics

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which contain digestive enzymes in the feed of sturgeons.

Soltani *et al*. (2019) reported that probiotics have beneficial effects on growth performance, resistance to diseases with increased innate immunity, reduction of pathogens in fish digestive tract. Microbial flora of aquatic animal is more fluid than terrestrial vertebrates and are highly sensitive to dietary change and is modified by life cycle changes, health status, rearing condition, ecological and environmental factors (Piazzon *et al*., 2017). Gut microflora have several functions that are beneficial to the health of the host by improving nutrient supply, promoting immune function, preventing the formation of colony by pathogens, energy balance, mucus integrity and function (Welker and Lim, 2011). The bacteria present in the aquatic environment affect the composition of the gut microbiota and vice versa. In this situation, probiotics must dominate (Ibrahem, 2013; Ghorbani Vaghei *et al*., 2019).

Bacillus probiotic is capable of producing antibiotics, amino acids, extracellular enzymes, dietary and bacterial effects for aquatic animals and is widely used in the aquaculture industry (Tabari *et al*., 2016). Probiotics include gram-positive and gram-negative bacteria and other microorganisms such as yeast and single-celled algae. Lactobacilli and bifid bacteria are widely used as probiotic commonly found in the intestines of healthy fish (Das *et al*., 2016). Lactic acid bacteria are the most important group of bacteria that are used in animal nutrition to improve growth, survival, and nutrition, to prevent gastrointestinal disorders, and to neutralize the anti-nutritional factors in the diet (Allameh *et al*., 2017).

The purpose of the present study was to determine the effects of native bacterial strains on growth performance, biochemical and immune indices, and intestine microflora of juvenile great sturgeon (*Huso huso*).

Materials and methods

Isolation of bacteria

To isolate the bacteria from the great sturgeon gut, sampling was done in a sterile condition. The intestine was cut in longitudinal direction and the content was removed. The inside of the intestine washed 3 times with physiological serum and homogenized. Then, the weighted material was transferred to sterile glass containers and physiological serum was added until a suitable dilution was achieved. It was serially diluted to 10^{-7}. Briefly, 0.1 mL of the prepared intestinal dilutions was poured onto de Man, Rogosa and Sharpe (MRS agar) medium. Plates were incubated at 30°C for 96 h at an anaerobic condition and then bacteria colony were counted as colony-forming unite (cfu/g). For purification, the samples were sub-cultured and biochemical tests, hot staining, catalase test, growth test (pH 4.4 and 9.6 and salinity 6.5%) at 10 and 45°C (in liquid MRS) were performed. Through 16SrRNA gene sequencing,

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lactic acid bacteria (LAB) *W. confusa* and *L. lactis* were molecularly identified in the great sturgeon intestine (Shenavar Masouleh *et al*., 2017).

White blood cells and total bacterial count in the intestine

At the end of experiment, fish were fasted for 24 h before blood sampling and 30% of fish per each tank were randomly chosen (Hallajian *et al*., 2011; Sayed Hassani *et al*., 2019). The blood samples were taken from the caudal vein using a 2 ml syringe and stored in non-heparinized tubes. For biochemical analysis, the blood samples were immediately centrifuged at 3000 g for 10 min at room temperature and then serum was separated and stored at −20°C until analysis (McPherson and Pincus, 2011). White blood cell (WBC) were measured by a spectrophotometer at 450 nm (UV/Vis-6505 N, Junway Company, England) using commercial kits (Pars Azmun Co. Ltd., Tehran, Iran). Lysozyme and compliment were measured by AutoAnalyzer Technicon (R.A.1000, Junway Company, England) using commercial kits (Pars Azmun Co. Model ISC and ILT., Tehran, Iran) described by Ellis 1990 and also IgM was determined through the nephelometric method using the Binding Site Kit (Yousefi Jourdehi *et al*. 2014; Sayed Hassani *et al*., 2019).

To determine viability and counting of bacteria in the intestine, 10 g of the intestine was weighted and the contents was washed 3 times using physiological serum and homogenized. Then, it was serially diluted to 10^{-7}. Briefly, 0.1 ml of the prepared intestinal dilution was poured onto trypton soy agar (TSA) medium and MRS agar medium. Plates were incubated at 30 °C for 96 h in anaerobic conditions and then colony-forming units (CFU/g) were counted (Merrifield *et al*., 2011; Sayed Hassani *et al*., 2019).

Research condition, preparation of diet and treatments

The research was conducted in the aquaculture department of the International Sturgeon Research Institute for two months. Initial mean weight (±SD) of fish was 79.44±3.18g. The study was performed with three experimental treatments with 3 replicates per each treatment, besides a control group without receiving probiotic. The diets were prepared through spraying a mixture of 50 ml of saline solution containing 150, 300 and 450 mg of 2 bacterial strains powder per kg of commercial pelleted diets (BioMar, France, composed of 42% crude protein, 18% lipid, 10% moisture, 10% ash, and 3.5% fiber) to make certain concentrations of 1.5×10^9 cfu/g (T_1), 3×10^9 cfu/g (T_2), 4.5×10^9 cfu/g (T_3). At random, 15 fish were stocked per each fiberglass tank (1m×1m×0.5m) containing 300 L freshwater. Effects of diets with different levels of probiotics (T_1, T_2 and T_3) on growth performance, immune indices (including lysozyme activity, alternative complement activity (ACH50), complements C_3 and C_4, monocyte, lymphocytes, neutrophils,

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and eosinophil), and intestinal microflora of great sturgeon juveniles was investigated. On average, dissolved oxygen, temperature, and pH of water were measured as 7.44±0.55 mg/L, 20.73±0.86°C, and 7.35±0.21, respectively.

Growth Performance analysis

At the end of study, all fish were fasted for 24 h and counted in each tank and then their average weight was determined. Growth performance indices including specific growth rate (SGR), food conversion ratio (FCR), condition factor (CF), and average daily growth (ADG) were determined as follows (Zare *et al*., 2017):

$WG= W_2- W_1$

$SGR= 100 (\ln W_2- \ln W_1)/ T$

$FCR= FO/ WG (g)$

$CF= \text{fish weight (g)}/ (\text{fish length cm})3\times100$

$ADG= (W_2- W_1)/ (W_1- T) \times 100$

Where:

ln= natural log, W1= initial weight (g), W2= final weight (g), T= time period in days, FO= feed offered (g), WG= weight gain, BW1= initial biomass weight, and BW2= final biomass weight. Survival rate was calculated at the end of the experiment: survival= (Nf/N0)×100; where N0 is initial number of fish and Nf is final number of fish.

Statistical analysis

This experiment was conducted based on a completely randomized design. All statistical analyses were performed using SPSS statistical package (version 16.0). One-way analysis of variance (ANOVA) and Duncan's multiple comparison tests were applied to identify significant variations at 0.95 confidence limits ($p<0.05$) between the treatments.

Results

As a result of adding two strains of bacteria to pelleted diet, there was no significant difference between treatments in terms of weight gain (g), biomass increase (g), specific growth rate, daily weight gain (g), and condition factors ($p>0.05$). Also, there was no significant difference in term of FCR among the control, T_1, and T_3 ($p>0.05$) (Table 1).

As to immune indices, the percentages of neutrophils in T_1 and T_2 significantly increased as compared to T_3 and control ($p<0.05$). The percentage of lymphocytes in the control group was higher than T_3, T_2, and T_1. However, there was no significant difference between T_1 and T_2 as well as between control and T_3 ($p>0.05$). Monocytes (%) in T_1 was higher than T_2, control, and T_3. There was no statistically significant difference between control and T_3 ($p>0.05$). Eosinophils (%) in the control fish was higher than the other treatments ($p>0.05$). IgM was in T_3, significantly more than other treatments and control ($p<0.05$). C_3 in T_1 and T_2 was significantly higher than the control and T_3 ($p<0.05$). C_4 was in T_1, T_2 and T_3 significantly more than control ($p<0.05$). Lysozyme (%) in T_1 was higher than T_2, control, and T_3. There

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was no significant difference between control and T_3 (P>0.05). ACH50 in T_2 and T_1 was significantly higher than control and T_3 (p<0.05) (Table 2).

Table 1: Effects of adding two bacterial strains on great sturgeon growth performance.

Growth indexes	Control group	Treatments		
		1 (150 mg/kg feed)	2 (300 mg/kg feed)	3 (450 mg/kg feed)
Initial weight (g)	80.22±1.37	83.34±0.11	78.83±2.52	79.05±1.27
Final weight (g)	287.03±16.95^a	291.85±20.06^a	274.70±32.10^a	281.19±28.56^a
Weight gain (g)	206.81±16.95^a	208.51±20.06^a	195.87±32.10^a	202.14±28.56^a
Biomass increase (g)	4305.5±254.36^a	4377.75±300.99^a	4120.55±481.56^a	4217.85±428.43^a
Condition factor	0.397±0.018^a	0.394±0.011^a	0.389±0.016^a	0.384±0.015^a
F.C.R	1.03±0.06^a	1.02±0.08^a	1.18±0.015^b	1.13±0.15^{ab}
S. G. R	1.51±0.05^a	1.49±0.08^a	1.48±0.15^a	1.50±0.14^a
Daily weight gain (g)	3.44±0.19^a	3.47±0.28^a	3.25±0.53^a	3.36±0.48^a

* Each value is mean ± SD. Different letters in each row mean significant difference (Duncan's multiple comparison tests, p<0.05).

Table 2: Effects of adding two bacterial strains on great sturgeon (*Huso huso*) immune indices.

Treatments	Parameters								
	IgM (mg/dl)	C_3 (mg/dl)	C_4 (u/l)	ACH50 U%)	Lysozyme Activity (u/ mL/min)	Lymphocytes (%)	Monocytes (%)	Eosinophils (%)	Neutrophils (%)
Control group	25.33 ± 2.51^a	17.66 ± 0.08^a	7.33± 1.52^a	110.66 ± 7.53^a	18.66± 1.52^a	79± 2^a	3.66± 1.15^{abc}	0.66± 0.57^a	16.66± 0.52^a
1	31.33 ± 3.21^b	22± 4.58^b	10.66 ± 2.08^b	124.33 ± 3.21^b	25.66± 5.03^b	73.612± 2.5^b	5.33± 0.57^{bc}	0.33± 0.57^a	20.66± 0.08^b
2	32± 1^b	9.33± 2.51^b	10.33 ± 2.08^b±	135.33 ± 7.37^c	28.33± 6.11^b	75± 1^a	4.33± 0.57^{ac}	0.33± 0.57^a	20.33± 0.57^b
3	32.66 ± 1.52^b	19± 2.64^b	8.33± 1.15^a	101.66 ± 5.03^d	19.33± 2.51^a	78.30.5± 0.57^a	3.33± 0.57^a	0.33± 0.57^a	18± 1^a

* Each value is mean ± SD. Different letters in each row mean significant difference (Duncan's multiple comparison tests, p<0.05).

The number of *Lactobacilli* in the intestinal mucosa of fish in the medium of MRS agar in T_1 was higher than T_2, control, and T_3, and also no significant difference was observed between control and other treatments (p>0.05).

In the medium of TSA, the number of aerobic and facultative anaerobic bacteria in the intestinal mucosa of fish in control was significantly more than T_1 and T_2 (p<0.05) (Table 3).

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Table 3: Effects of adding two bacterial strains on the number of lactic acid bacteria and aerobic and facultative anaerobic bacteria (Log-CFU/g) in the intestinal mucosa of great sturgeon in different media MRS agar and TSA.

Treatments	Culture medium	
	MRS agar (Log-CFU/g)	TSA (Log-CFU/g)
Control group	1.99 ± 0.18^{a}	4.75 ± 0.13^{a}
1	2.60 ± 0.54^{b}	4.22 ± 0.16^{b}
2	2.48 ± 0.43^{b}	4.16 ± 0.34^{bc}
3	1.77 ± 1.13^{a}	4.63 ± 0.14^{ac}

*Each value is mean ± SD. Different letters in each row mean significant difference (Duncan's multiple comparison tests, $p<0.05$).

Discussion

Growth performance

In the present study, there was no significant difference between treatments and control, in terms of weight gain (g) ($p>0.05$). Consistent with the present study, Soltani *et al*. (2016) after adding bacteria (*L. lactis*) to Persian sturgeon (*A. persicus*) diet did not report significant differences between treatments and control ($p>0.05$). Also, consistent with the research of Soltani *et al*. (2016), FCR in T_2 was significantly more than the other treated groups and control ($p<0.05$). In the line with the results reported by Soltani *et al*. (2016) there was no statistically significant difference in term of specific growth rate between treatments and control ($p>0.05$).

Despite the great effects of probiotics on the treated fish, they can increase immune responses (Irianto and Austin 2002). In vertebrates, it has been shown that maintaining and improving an active immunity can be energetically expensive as it is necessary to modification physiological activities (Soltani *et al*., 2019). Therefore, no significant difference between control and other treatments in terms of growth performance may be due to the mentioned issue.

Immune indices

In the present study, alternative complement activity (ACH50), complements C_3 and C_4, which are a complementary marker of fish's innate immunity status, in almost all treatments (T_1, T_2 and T_3, exception for ACH50 in T_3) were significantly more than control. The reason for the increase in complement composition is due to the WBC (Soltani *et al.* 2016) and this suggest that probiotics could modulate nonspecific immunity and increase tolerance of fish at high density and disease circumstances (Sayed Hassani *et al*., 2019).

In the present study, the number of white blood cells (except for eosinophils and lymphocytes) in T_1 and T_2, was significantly more than control and T_3 ($p<0.05$). In this relation, monocytes and neutrophils were significantly higher than control and T_3 ($p<0.05$) (Table 3). In this respect, it is consistent with the research conducted by other researchers from aspect of increase in the number of white blood cells as a result of the use of probiotics (Sadat Hosseini Madani *et al*.,

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2014; Das *et al*., 2016; Soltani *et al*., 2016; Asadi Khomami *et al*., 2017). White blood cells play an important role in immune system and are considered as the health indicator (Asadi Khomami *et al*., 2017). Since lymphocytes (B-lymphocyte and T-lymphocyte) are the most important cells that involve in the adaptive immunity and increase upon exposure to infection (Mousavi, 2012). Therefore, in the present study, the lower number of lymphocytes in the T_1, T_2 and T_3, especially in T_2 and T_3, can be attributed to the reduction of infection at the concentrations of probiotic used in the mentioned treatments. Consistent with the present study, a number of researchers (Venkatalakshima and Ebanser, 2015; Soltani *et al*., 2016; Sayed Hassani *et al*., 2019) have reported that probiotics significantly increased neutrophil count (p<0.05). Neutrophils are part of innate immunity, and extracellular microorganisms are captured and destroyed by neutrophils immediately after entering the body (Mousavi, 2012). Therefore, the introduction of probiotics through formulated diet into the body of fish may enhance readiness of the fish to deal with the undesirable microorganisms. Monocytes are a type of white blood cells involve in the immune system and release proteases from lysosomes. They also produce oxygen radicals and nitrogen oxides that eliminate infectious agents. Monocytes also produce cytokines that activate lymphocytes and stimulate the inflammatory process. Monocytes participate in the early stages of the immune response to phagocytosis (Pourgholam *et al*., 2017). Therefore, in the present study, due to the increase in monocyte in T_1 and T_2 compared to control and T_3, can conclude the role of two probiotic strains in enhancing the immune system. In line with the present study, in a study by Pourgholam *et al*. (2017) feeding on probiotics increased the number of white blood cells and monocytes of Siberian sturgeon compared to the control diet. They also pointed out that adding probiotics to diet can increase innate immunity to a greater extent than adaptive immunity.

There is a concordance between the present study and the study by Soltani *et al*. (2016) in terms of higher lymphocytes percentage in control in comparison with other treatments. In the present study, the eosinophils in the control was not significantly higher than other treatments (p>0.05) (Table 2). Soltani *et al*. (2016) reported that eosinophils in T_2 and T_3 was significantly more than T_1 and control (p<0.05). Since eosinophils are increased in allergic disease and parasitic infections in the blood, their lower percentage may represent the positive effects of probiotics.

In the present study, consistent with the results of other researchers (Soltani *et al*., 2016; Sayad Hasani *et al*., 2019; Kane *et al*., 2016; Alizadeh Rodposhti *et al*., 2017), IgM in treatments were significantly more than control (p<0.05) (Table 2). This immunoglobulin is one of the first humoral immune reactions and is an anti-pathogen, indicating of

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stimulating of lymphocyte population for IgM production as already reported by other researchers using some teleost fish (Sayed Hasani *et al*., 2019).

In a study by Soltani *et al*. (2016) in all treatments as with the present study (T_1 and T_2), the amount of lysozyme in the control was significantly lower than the mentioned treatments (p<0.05). Leukocytes have been reported as sources of lysozymes production (Soltani *et al*., 2016).

Impact on the number of lactic acid and aerobic and facultative anaerobic bacteria

As a result of adding the two strains of bacteria to great sturgeon diet, the count of *Lactobacilli* in the MRS agar medium in T_1 and T_2 was significantly higher than control and T_3 (p<0.05). Since the MRS agar medium is a selective medium for *Lactobacilli*, therefore this indicates the appropriate role of bacterial strains in the intestine. In the TSA medium, the count of aerobic and facultative anaerobic bacteria in control was significantly more than $T_1$and T_2 (p<0.05) (Table 3). Since the TSA medium is a kind of non-selective environment that provides sufficient nutrients for the growth of a wide range of microorganisms, this indicates that intestinal flora condition was worse in the control group.

Consistent with the present study, Alishahi *et al*. (2018) reported a significant increase in the number of intestine *Lactobacillus* as a result of adding two strains of probiotic separately *Lactobacillus plantarum* and *Lactobacillus bulgaricus* to the diet of common carp (*Cyprinus carpio*).

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