Iranian Journal of Fisheries Sciences 19(4) 2024-2038 2020
DOI: 10.22092/ijfs.2018.117847

Efficacy of dietary supplementation of *Bacillus licheniformis* and *Bacillus subtilis* probiotics and *Saccharomyces cerevisiae* (yeast) on the hematological, immune response, and biochemical features of Persian sturgeon (*Acipenser persicus*) fingerlings

Darafsh F.[1]; Soltani M.[2]; Abdolhay H.A.[3]*; Shamsaei Mehrejan M.[1]

Received: December 2017 Accepted: December 2018

Abstract

A total of 2,400 Persian sturgeon fingerlings weighing 3.50-3.80 g were fed a diet composed of but different amounts of D-pro probiotics (*Bacillus subtilis*, *B. licheniformis* and yeast *Saccharomyces cerevisiae*). Fish were distributed in fiberglass tanks (1.80 × 1.80 × 60 cm) each containing 200 fish for 2 months. This research was conducted in Beheshti Sturgeon Hatchery Center, North Iran in the summer of 2016. The mean temperature of the water (21±2℃), pH (7.5±0.5), and aeration and oxygen concentration (6±0.5 mg L^{-1}) were measured for 60 days. The calculated data were analyzed using one-way ANOVA test. Duncan test was used for comparing the means of the treatment. Results suggest that adding probiotics and yeasts to the diet had a significant impact on the percentages of hematocrit (PCV %), neutrophils and lysozyme (p<0.05). In addition, it was shown that the immunoglobulins in the T_1 and T_3 increased relatively to the control group. The amount of C_3 and C_4 complements were significantly increased by adding various sources of probiotics (p<0.05). Moreover, in terms of immune and biochemical parameters of the mucus sample, the interleukin 1 (T_1 and T_2) and lectin (T_1 and T_3) factors were improved. Also, in mucus samples, alkaline phosphatase and GPX all values measured in treated fish were lower than control group in various levels. These results show that application of two bacilli in combination form plus yeast may provide a better efficacy on the Persian sturgeon immune status.

Keywords: *Acipenser persicus*, *Bacillus licheniformis*, *Bacillus subtilis*, *Saccharomyces cerevisiae*, Hematology, Immunology

1-Department of Aquaculture and Fisheries, Science and Research Branch, Islamic Azad University, Tehran, Iran.

2-Department of Aquatic Animal Health, Faculty of Veterinary Medicine, University of Tehran, Tehran, Iran.

3-Iranian Fisheries Sciences Research Institute (IFSRI), Agricultural Research, Education and Extension Organization (AREEO), Tehran, Iran

*Corresponding author's Email: hossein_abdolhay@yahoo.com

Introduction

Sturgeons are quite desired in terms of biodiversity and genetic diversity since they are able to survive for a long time. These fish are of crucial economic importance because of the caviar they produce and their meat (Pourkazemi, 2006). Among sturgeons, *Acipenser persicus* is a valuable species, which was first seen in the Ural River (Khodorevskaya and Novikova, 1995).

Probiotics are nowadays known as appropriate and safe additives in diets. Many probiotic species belong to a large family of bacteria named lactobacillales or lactic acid bacteria (LAB). Other probiotics are yeasts including *Saccharomyces* and bacteria such as *Enterococcus faecium* and *Bacillus subtilis* (Valeur *et al.*, 2004).

Probiotics contain live and/or dead microbial cells. When added to diets, the probiotics can have positive impacts on mechanisms such as improvement of the microbial populations or improvement of microbial growth (Balakrishna and Keerthi, 2012).

Probiotics play an important role in the health and improvement of growth of fish and can stimulate the specific and non-specific immune systems. Probiotics that contain one or multiple species of yeast or bacterium are able to stimulate lysozyme and phagocytic activities and increase the function of fish cytokines (Allameh *et al*., 2017). Notably, numerous factors such as resource, species, the dosage and duration of consumption of probiotics affect their immunity-related activities (Allameh *et al.*, 2017). Using lactobacillus as additives has lead to a strong and appropriate immune response against microorganisms (Salaghi *et al.*, 2013).

Licheniformis bacillus is a part of the subtilis family along with *B. subtilis* and *B. pamilus.* They play important roles in the production of antibiotics, biosurfactants, alkaline amylase, and proteins (Smitha and Bhat, 2013). Since *Bacillus subtilis* is able to produce compounds with antimicrobial properties such as antifungal lipopeptides, they are effective when it comes to fungal pathogens (Ongena and Jacquea, 2007). Since this bacterium is a resistant spore-forming bacterium, it is one of the best candidates in the biological control process (Fickers *et al*., 2008).

Saccharomyces cerevisiae yeast is one of the most important industrial yeasts for producing biochemical compounds, recombinant proteins, and single-cell proteins. The performance of *Saccharomyces cerevisiae* depends on its strain (Fietto *et al.*, 2004). Saccharomyces var. boulardii yeast seems to have an impact on the metabolism of *Oncorhynchus mykiss* and increase lipid and pigmentation of the muscle (Aubin *et al*., 2005).

Given the importance of Persian sturgeon, *A. percsicus,* in the economy of the fish meat and caviar production industry and also considering the role of probiotics in the diets of different fish, the present study aimed to examine the effect of oral consumption of licheniformis and *B. subtilis* probiotics and *S. cerevisiae* yeast on the hematologic, immunological, and

biochemical parameters of Persian *A. persicus*.

Materials and methods

Fish

A. persicus fingerlings weighing 3.5-3.8 g obtained from a sturgeon hatchery center were used. Fish were randomly distributed in 1.8 × 1.8× 0.6 m fibreglass tanks with 200 fish/tank. Fish were acclimatized for 14 days to new conditions and were fed Coppens Feed (Germany) in the summer of 2016. Water quality parameters included temperature 21±2 °C, pH 7.5±0.5, dissolved oxygen>6 mg L^{-1}, nitrite <0.1 mg L^{-1} and unionized ammonia <0.01 mg L^{-1}. The water supply source was a combination of well and river water, 60% of well water and 40% of river water, with a total flow rate of 0.20 liters per second and was distributed between baths in completely identical conditions.

Probiotic and yeast

Commercial probiotic named Dipro contains *B. licheniformis* and *B. subtilis (*each at1.6×10^{12} cfu kg^{-1}) (Takgen Zist Lmt, Iran) and *S. cerevisiae* at 5 g kg^{-1} feed (Takgen Zist Lmt, Iran) was used.

Experimental design

The first and second groups were fed with Dipro probiotic (DP) and *S. cerevisiae* (SC) each at 5 g kg^{-1} feed. The third group was fed with the mixture of DP +SC each at 5 g kg^{-1} feed. The fourth group was considered as control fish using basal feed. Each treatment was considered in three replicates with 200 fish/replicate. Gelatin at 30 g L^{-1} at 51°C was used as the coater.

Sampling

At the end of the trial, feeding was stopped for 24 hours and blood samples were taken from 150 fish in each treatment after fish were anaesthetized with clove oil at 75 ppm. The obtained heparinized blood samples were processed for hematological parameters, while the un-heparinized blood samples were centrifuged before sera separation for immuno-biochemical assays as described below. Also, samples of gut mucus were collected from 6 samples in each treatment in sterile Eppendorf tubes and were immediately frozen at -70 ˚C until examined.

Hematological assays

The hematological indices consisting of red blood cell (RBC) count, white blood cell (WBC) count, hematocrit percentage (PCV %), hemoglobin concentration (HB), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC) and mean corpuscular volume, or mean cell volume (MCV) were carried out using methods described by Klontz (1994). The leukocyte differential count was carried out using the blood smears under compound light microscope at x 400. The blood smears were first dried, fixed with methanol and stained with Giemsa stain prior to the examination. (Klontz, 1994).

Immuno-biochemical assays

Alkaline phosphate (ALP) was measured using an Auto-analyzer device (Belgium Eurolyser). Serum Total protein was obtained using an Auto-analyzer (BT-1500, Biotecnica, Italy) at 520 nm using the Biuret method (Hoseinifar *et al*., 2011). Lysozyme level was measured according to Ellis (1990) using *Micrococcus lysodeikticus* (Sigma, USA), and lysozyme from egg white lyophilized powder (Sigma) was used for drawing the standard curve. The values of C_3, C_4, and CH_{50} were measured according to Stolen *et al.* (1990). In addition, Easbiopharm kit (Hangzhou *Eastbiopharm Co., Ltd* , USA) was used for detection of lectin and interleukin values in sera samples. Glutathione peroxidase was measured with method Peglia and Valentine (1976).

Catalase activity was determined by measuring the decrease of H_2O_2 concentration at 410 nm according to Koroliuk *et al.* (1988). Moreover, immunoglobulin M (IgM) content was estimated according to the method described by Siwicki and Anderson (1993). High-sensitive CRP (hs-CRP) was calculated using methods described by Kodama *et al.* (2003). Finally, Superoxide dismutase activity was determined according to Kostiuk *et al.* (1990).

Statistical model and the experimental design

A completely randomized design with 4 treatments and three replications was used. The obtained data were analyzed using the one-way ANOVA test. Duncan test was used for comparing the means of the treatment. A 5% significance level was determined for comparing the means of the treatments.

Results

Immuno-biochemical parameters

The immuno-biochemical values results are shown in Table 1.

Table 1: Immunological and biochemical indexes of Persian sturgeon serum fed with bacilli probiotics and yeast for 2 months. DP= *Bacillus subtilis* + *Bacillus licheniformis*, *SC*=*Saccharomyces cerevisiae*, DP+SC= *B. subtilis* + *B. licheniformis*+ *S. cerevisiae.*

Index	Unit	Treatments			
		DP	SC	DP+SC	Control
Lysozyme	µg ml^{-1}	23.33±3.83^{b}	20.5±3.14^{c}	28±4.38^{a}	17.66±1.75^{d}
Total immunoglobin	mg ml^{-1}	11.83±0.42^{b}	10.72±0.29^{c}	14.98±0.51^{a}	10.65±0.22^{d}
Interleukin-1	mg ml^{-1}	12.46±1.76^{b}	12.05±2.89^{c}	14.1±2.49^{a}	11.42±1.74^{d}
Lectin	µg ml^{-1}	85.10±13.48^{a}	30.92±5.59^{c}	64.45±6.26^{b}	28.48±5.73^{d}
Catalas	U ml^{-1}	63.16±3.97^{c}	87.33±12.53^{a}	71.16±4.91^{b}	48.50±12.06^{d}
Superoxide dismutase	U ml^{-1}	156±33.34^{a}	139.96±27.87^{c}	145.60±29.67^{b}	136.18±13.74^{d}

* Different letters indicate significant differences (p<0.05).

As can be noticed, the blood sample in T_3 had the highest level of lysozyme enzyme (28 µg mL^{-1}) and the differences between this treatment and others were significant (p<0.05). The lowest level of lysozyme to control was in the blood serum sample of T_2 (20.5 µg mL^{-1}).

In addition, the blood serum sample of T_3 had the highest level of total immunoglobin (14.98 mg mL^{-1}) and it had a significant difference with the control and the other two treatments (p<0.05). However, there was no considerable difference between T_2 and control.

The interleukin in the blood serum was increased when adding various sources of probiotics. This increase was most exponential for T_3, in which samples increased two different sources of probiotics, i.e. D-pro and SC. However, this increase was statistically significant compared to that of T_2. An increase was also observed in T_1, but this increase was less considerable than the increased in T_3; however, there was no significant difference between the control and T_2 (p>0.05). Adding only one source of probiotics to the diet, i.e. SC, led to an increase in the level of interleukin-1 in the blood serum.

The level of lectin in T_1 increased significantly compared with the control treatment. It is noteworthy that when both D-pro and yeast were added as probiotics to the diets, lectin underwent a 126.3% increase. The results obtained by comparing the means of various treatments were somehow different for the catalase enzyme. For example, in the case of T_1, where D-pro was added to the diet as a probiotic, the level of this enzyme increased and this increase was statistically significant (p<0.05). The level of this enzyme increased to 87.33 U mL^{-1} in T_2 when yeast was added to the diets. It has to be noted that in T_3, the level of catalase was significantly increased to 46.72%. The highest level of superoxide dismutase was observed in the blood serum sample of Treatment 1 (156.20 U mL^{-1}). Compared to the control and other treatments, the increase in the level of SOD was significant (p<0.05). The lowest level of SOD was observed in T_2, which was not statistically significant compared to the control (p>0.05). In T_3, the level of this enzyme had a 6.92% increase (to 145.60 U mL^{-1}) relative to the control. The changes of the immune factors and biochemical indexes in the blood serum samples taken from Persian sturgeon fingerlings.

Table 2 illustrates the comparison made between the means of the biochemical indexes and mucosal immune factors of Persian sturgeon fingerlings.

Table 2: Immunological variables measured in mucus of Persian sturgeon fed with bacilli probiotic and yeast for 2 months. DP= *Bacillus subtilis + Bacillus licheniformis, SC= Saccharomyces cerevisiae*, DP+SC= *B. subtilis + B. licheniformis+ S. cerevisiae*.

Index	Unit	Treatments			
		DP	SC	DP+SC	Control
C_3	μg L^{-1}	30.01±0.01^{c}	44.1±0.01^{b}	50.01±0.01^{a}	19.01±0.01^{d}
C_4	μg L^{-1}	26.01±0.01^{b}	17.01±0.01^{c}	30.34±0.01^{a}	10.01±.01^{b}
CH_{50}	U ml^{-1}	36.1±0.01^{c}	46.75±0.01^{b}	53.1±0.01^{a}	24.01±0.01^{d}
IL 1	mg L^{-1}	30.46±0.06^{b}	30.63±0.06^{a}	27.01±0.01^{c}	26.43±0.06^{d}
Lectin	μg ml^{-1}	101.30±0.01^{a}	77.86±0.06^{c}	83.70±0.01^{b}	76.20±0.01^{d}
GPX	U ml^{-1}	4.01±0.01^{c}	5.2±0.01^{b}	3.5±0.01^{d}	6.41±0.01^{a}
CRP	mg dl^{-1}	0.5±0.001^{a}	0.1±0.001^{a}	0.5±0.001^{a}	0.1±0.001^{a}
Alkaline phosphate	U ml^{-1}	8.02±0.01^{d}	10.01±0.01^{b}	8.6±0.01^{c}	14.01±0.01^{a}
Serum Total protein	mg dl^{-1}	0.6±0.001^{a}	0.5±0.101^{a}	0.41±0.001^{a}	0.2±0.001^{a}

* Different letters indicate significant differences (p<0.05).

As can be seen, the addition of various sources of probiotics led to the increase of C_3 and this increase was statistically significant (p<0.05). The most exponential increase was observed in T 3. Therefore, the level of C_3 in T_3 had a significant difference with that in the control and the other two treatments. The second highest level of C_3 was that of T_2; it underwent a 131.98% increase, which was statistically significant (p<0.05). The variations of the level of C_4 with the addition of various sources of probiotics were to some extent similar to that of C_3. In the control, the level of C_4 was 10.01 μg L^{-1} and adding probiotics led to a significant increase in this level. In this regard, the maximum increase was observed in T_3, where both sources of probiotics were received by the samples.

The level of interleukin-1 in the mucus was equal to 26.43 mg mL^{-1} based on the control diet. This level was increased to 30.46 mg mL^{-1} in the T_1 after D-pro was added to the diet (a 15.25 % increase). In T_2, with the addition of the yeast to the diet, the variations were the same without any considerable change and there was significant difference between T_1 and T_2 in this regard. It is of note that the level of interleukin in T_3 was reduced when adding both sources of probiotics; however, this reduction was statistically significant.

The results obtained from comparing the means for the level of lectin show that adding D-pro causes a significant increase; whereas the addition of yeast as the only source of probiotics in T_2 led to a slight increase in the level of

lectin. The analysis results showed significant difference between T_2 and the control (p<0.05). The level of lectin was increased in T_1 and T_3.

The level of the glutathione peroxidase enzyme in the control was equal to 6.41 U ml^{-1}. This level was decreased in T_1 with the addition of D-pro complement; this decrease was significant (p<0.05). In T_2, when *Saccharomyces cerevisiae* yeast was added to the diet, the level of glutathione peroxidase was significantly decreased (p<0.05). In T_3, the reduce was more exponential (3.5U mL^{-1}) and significantly different in the control and other treatments.

Acute phase protein was equal to 0.1 mg dl^{-1} in the control. Notably, a considerable procedure was observed in the level of acute phase protein when various sources of probiotics were added; meaning that the levels of acute phase protein were similar in T_1 and T_3, not to mention that the level of acute phase protein had not a significant raise relative to the control. Alkaline phosphate in the mucus sample underwent reduce as various sources of probiotics were added. In the control, alkaline phosphate was equal 14.01 U mL^{-1} which was decrease to 8.02 U mL^{-1} in T_1 which was statistically significant (p<0.05). In T_2, where *Saccharomyces cerevisiae* yeast was added as the probiotic, the level of alkaline phosphate was reduced to 10.01 U mL^{-1} and this decrease was the lowest among all of the treatments. There was a significant difference between this treatment and the control as well as the other treatments (p<0.05). In T_3, the level of alkaline phosphate was decreased to 8.6 U mL^{-1} compared with the control. It seems that adding *Saccharomyces cerevisiae* yeast as a probiotic led to the lowest decrease in the level of alkaline phosphate compared with adding D-pro.

As presented in Table 2, serum total protein in the mucus sample was equal to 0.2 mg dL^{-1} in the control treatment. T_1 and T_2 were similar in terms of the rise of the level of total protein in the mucus sample; the level of total protein in the mucus sample was equal to 0.6 g L^{-1} and 0.5 mg dL^{-1}. In T_3, where two sources of probiotics were added, the reduction in the level of total protein was the lowest amount in comparison with T_1 and T_2.

Hematological parameters

Table 3 and Table 4 show the results obtained from comparing the mean of immunocompetent cell population and hematological indexes of blood-based on the conditions of various treatments.

Table 3: Immunocompetent cell population in of Persian sturgeon fingerlings fed Bacillus probiotics and yeast for two months. DP= *Bacillus subtilis* + *Bacillus licheniformis, SC= Saccharomyces cerevisiae*, DP+SC= *B. subtilis* + *B. licheniformis*+ *S. cerevisiae.*

Value	Treatments			
	DP	**SC**	**DP+SC**	**Control**
WBC (10^3 cells μl^{-1})	22.21 ±1.32^b	20.62 ±1.35^c	23.23± 1.^{a}	20.2±1.1^d
Neutrophil (%)	22.53±1.21^a	22.21±1.38^b	23.44±1.04^c	21.45 ±1.1^d
Lymphocyte (%)	68.56 ±1.32^a	67.7± 1.45^b	68.79 ±1.65^a	67.44±2.1^b
Monocyte (%)	3.7± 1.21^b	3.78± 1.03^a	3.7 ±1.2^b	3.7±1.2^b
Eosinophil (%)	5.21 ±1.01^c	6.31± 0.68^b	4.07 ±1.55^d	6.41±1.73^a

* Different letters indicate significant differences (p<0.05).

Table 4: Hematological indexes in of Persian sturgeon fingerlings fed Bacillus probiotics and yeast for two months. DP= *Bacillus subtilis* + *Bacillus licheniformis, SC= Saccharomyces cerevisiae*, DP+SC= *B. subtilis* + *B. licheniformis*+ *S. cerevisiae.*

Hematological index	Treatments			
	DP	**SC**	**DP+SC**	**Control**
RBC (10^4 cells μl^{-1})	70±10.54^b	68.67± 11.22^c	73± 12.34^a	66.32 ±14.54^d
HB (g dl^{-1})	5.40±0.3^c	5.66±0.258^b	5.73±0.31^a	5.25±0.30^d
PCV (%)	25.66±1.75^b	24.76±1.78^c	26.50±1.05^a	22.66±1.21^d
MCV (fI)	231.43±43.9^d	246.09±49.2^a	235.48±49.44^c	238.64±62.04^b
MCHC (Pg dl^{-1})	33.33±7.73^a	33.33±13.33^a	31±12.4^b	33.33±17.33^a
MCH (Pg)	77.14±14.66^d	82.03±16.40^a	78.49±16.48^c	79.54±20.68^b

* Different letters indicate significant differences (p<0.05).

The number of white blood cells was significantly increased in T_3, where a combination of yeast and D-pro was added (a 13.04% increase). It was the same for the red blood cells; in the sense that the number of red blood cells was increased in Treatments 1 and 2 (p<0.05), also it was increased in T_3. It is worth mentioning that addition of D-pro in T_1 and T_3 led to an increase in the number of RBC (p<0.05).

In T_1, hematocrit percentage (PCV %) was increased when D-pro was added. In comparison to the control treatment, hematocrit percentage was increased in T_2 (p<0.05). The hematocrit percentage was increased in T_3 with the addition of both sources of probiotics and this raise was equal to 8.38%. The MCV was decreased in all of the treatments (p<0.05) and the increase was observed in T_2, where SC was added. With the addition of SC, the MCH was increased in the T_2 . With the addition of D-pro and SC in T_3, the MCHC was decreased and this decrease was statistically significant (p<0.05).

The level of neutrophils was increased in all treatments relative to the control (p<0.05); In T_1, where D-pro was added, lymphocytes had a significant increase (p<0.05). In all treatments, monocytes were similar with the addition of probiotics and the differences between the control treatment and the other three were not significant. The levels of eosinophil were reduced in all treatments compared with the control. An eosinophil reduction is observed in conditions where the fish is influenced by acute stress, such as stress on the fish during a blood sampling due to secretion of substances including adrenaline, glucocorticosteroid or epinephrine.

Discussion

There are some studies that confirm the positive effects of probiotics, prebiotics, immunostimulants, and vitamins on different aquatic species (Hoseinifar *et al.*, 2011; Chelladurai *et al.*, 2013; Mohapatra *et al.*, 2014; Kane *et al.*, 2016).

Obviously, these probiotics boost the immune system to defend the body against pathogenic organisms. Also, they neutralize the negative effects of antibiotics and chemotherapy agents. The compounds created by bacteria help reinforce the immune response of fish, lobster, and white shrimp (Akter *et al.*, 2015;). Probiotics such as *Lactobacillus plantarum* have shown established properties for improving the immune system, helping the body defend itself better against pathogenic microorganisms such as streptococci and lactococci, and for improving growth by stimulating the immune system and increasing its efficiency (Kane *et al.*, 2016; Allameh *et al.*, 2017). Lysozyme is a protein that has been found in various vertebrate species, including fish. Most of the antimicrobial activities of this protein are against gram-positive bacteria and some of the gram-negative bacteria as well. Lysozyme breaks the beta 1, 4-glycosidic bond between N-acetylmuramic and N-acetylglucosamine in the peptidoglycan layer in cell walls of bacteria (Balcázar *et al.*, 2007). In addition, it activates complements and increases the phagocytic activity in the serum (Ahmadifar *et al.*, 2011*).* The level of lysozyme reduces in fertilized eggs and this reduction continues until larvae are 10-days old. From this point onward, the level of lysozyme increases. It is quite the opposite for the level of C_3 complement (Abdollahi *et al.*, 2016). The immunological indexes change when probiotics are added to the diets (Imanpoor and Roohi, 2015). This behavior was observed in the present study when the *B. licheniformis* and *B. subtilis* probiotics were added to the diet; suggesting that by adding probiotics, the level of lysozyme in the blood serum was significantly enhanced. The increase in lysozyme following the addition of *B. licheniformis* and *B. subtilis* bacteria, as probiotics, to the ration can cause elevations in antimicrobial activities and balance the sturgeon fingerlings' intestinal micro-flora. This behavior is accompanied by the macrophagic activities and harmful microbes' elimination of the digestive tract. Kane *et al.* (2016) state that lysozyme rates significantly increase with the addition of *Lactococcus lactis* probiotics to the ration administered to the Iranian sturgeon fingerlings.

Immunoglobulins are among the most important humoral immune factors in fish that play a key role in preventing harmful microorganisms (Akter *et al.*, 2015). It has been evidenced that probiotics are able to increase the innate and specific immune responses of fish by improving the production of total immunoglobulins (Khan *et al.*, 2016). In the present study, it has been shown that adding probiotics such as *B. licheniformis* and *B. subtilis*, and the *S. cerevisiae* yeast

significantly increased the level of total immunoglobulins. This finding supports the specific and inherent immunity response of the Iranian sturgeon fingerlings. However, in addition to the type of probiotic added, the applied concentration of the stimulant and management methods in fish breeding are also key factors that affect the immune responses of fish. In the research conducted by Taati *et al.* (2011), it was reported that the levels of IgM in the blood serum of *Huso Huso* (beluga) that had received a diet containing 1% mannan-oligosaccharide prebiotic were significantly higher than that of the fish in the control group. In this connection, Khan *et al.* (2016) reported that adding *Lactobacillus plantarum* to the diets significantly increases the level of immunoglobulin G, Serum total protein, alkaline phosphatase, and cells such as leukocytes and monocytes. Chelladurai *et al.* (2013) have also reported that adding prebiotics to the diets increases the level of immunoglobulins.

Also, compliments are among the non-specific immune factors that have a significant impact on the immune response of fish. It has been frequently reported that using immunostimulants increases the activities of complements (Abdollahi *et al.,* 2016). It seems that the improvement of the immunity system occurs in sturgeons subsequent to the increase in C_3 and C_4 complements; and this improvement is per se highly effective on their general health.

In the present study, it was observed that the levels of C_3, C_4 complements, and CH_{50} were significantly increased when various sources of probiotics, including licheniformis and *B. subtilis* bacteria and *S. cerevisiae* yeast, were added to the diets. This result complies with the results obtained by other scholars (Abdollahi *et al.,* 2016). Mucus is one of the innate immune mechanisms that is always present and is continuously produced when dead tissues of skin are removed. Mucus prevents adhesion of pathogens that in turn helps the defence system. In this context, fish mucus is an important source of substances involved in the non-specific immune system, including lysozyme, immunoglobulins, complementary proteins (complementary agents), lectins, proteolytic enzymes, c-reactive proteins, other proteins, and antibacterial lipids (Subramanian *et al.*, 2007). In the present study, a drop was observed in the level of glutathione peroxidase enzyme following the addition of probiotics. This result does comply with the results obtained from some of the other investigations. In some studies, a drop was observed in the activity of enzymes and the products of oxidation of superoxide dismutase and catalase in *Litopenaeus stylirostris* and *Mycteropercarosacea* receiving a diet that is rich with *Debaryomyces hansenii* and *Pediococcus acidilactici* (Pereira, 2014). Moreover, in the research conducted by Tovar-Ramírez *et al.* (2010) on *Lates calcarifer*, it was concluded that although using yeast does not have an impact on the catalase enzyme, it reduces the activity of

glutathione peroxidase; which complies with the results of the present study. In *Litopenaeus stylirostris*, the activity of glutathione peroxidase has increased when the samples received a diet containing *B. subtilis* (Shen *et al.*, 2010). Li *et al.* (2015) have shown that all of the immunological parameters, including phagocytic activities, superoxide dismutase, catalase, and the activity of phenoloxidase, significantly are improved when *B. cereus* and *B. subtilis* are added to the diets. Thus, using a combination of probiotics stimulates the non-specific immune response and improves growth and resistance to pathogens (Li *et al.*, 2015).

Hematological indexes

Hematological parameters, as the indication of the health status of the fish, are clearly influenced by their physiological status (Harikrishnan *et al.*, 2010). Numerous studies have reported that when probiotics are added to the diets, an optimum condition is obtained for hematological parameters including the number of white blood cells, number of red blood cells, cellular volume, and hemoglobin (Olayinka and Afolabi, 2013). Therefore, blood parameters act as valuable measures for evaluating fish's level of health (Neissi *et al.*, 2013). The findings of the present study have shown that adding probiotics to the diets of the samples has a statistically significant impact on the WBC count, RBC count, PCV, and percentage of neutrophils. The results of this research regarding the improvement of blood indexes comply with the results of other studies (Chelladurai *et al.*, 2013). Chelladurai *et al.* (2013) showed that adding *L. acidophilus*, as a probiotic, to the diets significantly increases blood parameters including red blood cells, white blood cells, serum protein, glucose, cholesterol, and mineral ions such as magnesium, calcium, and chlorine. Blood parameters probably become more efficient because the fish are under less stress. Also, because they consume probiotics, they become more resistant to infections. Faeed *et al.* (2016) showed that out of all the blood parameters, only hematocrit and MCV are significantly increased when *Enterococcus faecium* is added to the diets as a probiotic.

Generally, the results of this research indicate that adding D-pro probiotic and *S. cerevisiae* yeast to the diet of Persian sturgeon fingerlings can improve some of the hematological parameters and this improvement is more considerable where D-pro was added (T_3, T_1). Studying immune factors and biochemical indexes in mucus showed some improvement in interleukin (T_1, T_2) and lectin (T_1, T_3). About immune factors and biochemical indexes in the blood serum samples, different factors separately improved the efficiency of T_1, T_2 and T_3, receiving D-pro, *S. cerevisiae* yeast, and a combination of D-pro and SC yeast.

Acknowledgements

The researchers would like to express their sincere gratitude to the chairman of Shahid Beheshti Sturgeon Stocks Reproduction and Reconstruction Center as well as others who

contributed to the conduction of this project.

References

Abdollahi, R., Heidari, B. and Aghamaali, M., 2016. Evaluation of lysozyme, complement C_3, and total protein in different developmental stages of Caspian kutum (*Rutilus frisii kutum* K.). *Archives of Polish Fisheries*, 24(**1**), 15-22. DOI: 10.1515/aopf-2016-0002

Ahmadifar, E., Akrami, R., Ghelichi, A. and Mohammadi Zarejabad, A., 2011. Effects of different dietary prebiotic insulin levels on blood serum enzyme, hematologic and biochemical parameters of great sturgeon (*Huso huso*) juvenile. *Comparative Clinical Pathology*, 20(**5**), 447-451.

Akter, N.M., Sutriana, A., Talpur, A.D. and Hashim, R., 2015. Dietary supplementation with mannan oligosaccharide influences growth, digestive enzymes, gut morphology, and microbiota in juvenile striped catfish Pangasianodon hypophthalus. *Aquaculture International*, 24(**1**), 127-144. DOI: 10.1007/s10499-015-9913-8

Allameh, S.K., Noaman, V. and Nahavandi, R., 2017. Effects of probiotic bacteria on fish performance. *Advanced Techniques in Clinical Microbiology*, 1(**2:11**), 1-5.

Aubin, J., Gatesoupe, F.J., Quentel, C., Labbé, L. and Forraz, M., 2005. Ofimer probiotic study on rainbow trout. III. Flesh quality assessment of rainbow trout (*Oncorhynchus mykiss*) submitted to probiotic treatment with Saccharomyces cerevisiae var. boulardii. In B. Howell & R. Flos (Eds.), Lessons from the past to optimise the future, aquaculture Europe (pp. 115-116). Belgium: European Aquaculture Society.

Balakrishna, A. and Keerthi, T.R., 2012. Screening of potential aquatic probiotics from the major microflora of guppies (*Poecilia reticulate*). *Frontiers of Chemical Science and Engineering*, 6(**2**), 163-173. DOI: 10.1007/s11705-012-1283-4

Balcázar, J.L., de Blas, I., Ruiz-Zarzuela, I., Vendrell, D., Gironés, O. and Muzquiz, J.L., 2007. Enhancement of the immune response and protection induced by probiotic lactic acid bacteria against furunculosis in rainbow trout (*Oncorhynchus mykiss*). *FEMS Immunology and Medical Microbiology*, 51(**1**), 185-193. DOI: 10.1111/j.1574-695X.2007.00294.x

Chelladurai, G., Jebaraj, F. and Rathinasami, N., 2013. Protective effect of probiotic diets on haematobiochemical and histopathology changes of *Mystus montanus* (Jerdon 1849) against *Aeromonas hydrophila*. *Journal of Coastal Life Medicine*, 1(**4**), 259-264. DOI: 10.12980/JCLM.1.2013c1088

Ellis, A.E., 1990. Lysozyme assays. In: Techniques in fish immunology, ed. J.S. Stolen, T.C. Fletcher, D.P. Anderson, B.S. Roberson, and W.B. Van Muiswinkel, New Jersey, USA: SOS Publications. pp. 101-113.

Faeed, M., Kermanshahi, R., Pourkazemi, M., Darboee, M. and Karsidani, H., 2016. Effect of the probiotic *Entrococcus faecium* on hematological and non-specific immune parameters and disease resistance in zander (*Sander lucioperca*). *Iranian Journal of Fisheries Sciences*, 15(**4**), 1581-1592.

Fickers, P., Lecle`re, V., Guez, J., Be´chet, M., Coucheney, F., Joris, B. and Jacques, Ph., 2008. Temperature dependence of mycosubtilin homologue production in *Bacillus subtilis* ATCC6633. *Research in Microbiology*, 159 (**6**), 449-457.doi: 10.1016/j.resmic.2008.05.004

Fietto, J.L.R., Araujo, R.S., Valadao, F.N., Fietto, L.G., Brandao, R.L., Neves, M.J., Gomes, F.C.O., Nicoli, J.R. and Castro, I.M., 2004. Molecular and physiological comparisons between *Saccharomyces cerevisiae* and *Saccharomyces boulardii*. *Canadian Journal of Microbiology*, 50(**8**), 615-621. DOI: 10.1139/w04-050

Harikrishnan, R., Balasundaram, C. and Heo, M.S., 2010. *Lactobacillus sakei* BK19 enriched diet enhances the immunity status and disease resistance to streptococcosis infection in kelp grouper, *Epinephelus bruneus*. *Fish and Shellfish Immunology*, 29(**6**), 1037-1043. DOI: 10.1016/j.fsi.2010.08.017

Hoseinifar, S.H., Mirvaghefi, A., Merrifield, D.L., Amiri, B.M., Yelghi, S. and Bastami, K.D., 2011. The study of some haematological and serum biochemical parameters of juvenile beluga (*Huso huso*) fed oligofructose. *Fish Physiology and Biochemistry*, 37(**1**), 91-96.

Imanpoor, M.R. and Roohi, Z., 2015. Influence of primalac probiotic on growth performance, blood biochemical parameters, survival and stress resistance in the Caspian roach (*Rutilus rutilus*) fry. *Turkish Journal of Fisheries and Aquatic Sciences*, 15(**4**), 917-922. DOI: 10.4194/1303-2712-v15_4_15

Kane, A.M., Soltani, M., Ebrahimzahe-Mousavi, H.A. and Pakzad, K., 2016. Influence of probiotic, *Lactobacillus plantarum* on serum biochemical and immune parameters in vaccinated rainbow trout (*Oncorhynchus mykiss*) against streptococcosis/lactococosis. *International Journal of Aquatic Biology*, 4(**4**), 285-294.

Khan, M.W., Priyamvada, S., Khan, S.A., Khan, S., Gangopadhyay, A. and Yusufi, A.N.K., 2016. Fish/flaxseed oil protect against nitric oxide-induced hepatotoxicity and cell death in the rat liver. *Human and Experimental Toxcicology*, 35(**3**), 302-311. DOI: 10.1177/0960327115586207

Khodorevskaya, R.P. and Novikova, A.S., 1995. Status of Beluga Sturgeon, *Huso huso*, in the Caspian Sea. *Journal of Ichthyology*, 35(**9**), 59-68.

Klontz, G.W., 1994. Fish hematology. In: Techniques in fish immunology, Stolen, J. S.; Flecher, T. C.; Rowely,

A. F.; Zelikoff, T. C.; Kaattari S. L. and Smith S. A. (Eds.). Vol. 2, SOS Publications, USA. pp. 121-132.

Kodama, H., Matsuoka, Y., Tanaka, Y., Liu, Y., Iwasaki, T. and Watarai, S., 2003. Changes of C-reactive protein levels in rainbow trout (*Oncorhynchus mykiss*) sera after exposure to anti-ectoparasitic chemicals used in aquaculture. *Fish and Shellfish Immunology*, 16 (**5**), 589-597.

Koroliuk, M.A., Ivanova, L.I., Maiorova, I.G. and Tokarev, V.E., 1988. A method of determining catalase activity. *Lab Delo*, 1, 16-19.

Kostiuk, V.A., Potapovich, A.I. and Kovaleva, Zh.V., 1990. A simple and sensitive method of determination of superoxide dismutase activity based on the reaction of quercetin oxidation. *Voprosy Meditsinskoi Khimii*, 36(**2**), 88-91.

Li, J., Xu, Y., Jin, L. and Li, X., 2015. Effects of a probiotic mixture (*Bacillus subtilis* YB-1 and *Bacillus cereus* YB-2) on disease resistance and non-specific immunity of sea cucumber, *Apostichopus japonicus* (Selenka). *Aquaculture Research*, 46(**12**), 3008–3019. DOI: 10.1111/are.12453

Mohapatra, S., Chakraborty, T., Ashisa, K.P., Kurchetti, P.P. and Kedar, N.M., 2014. Beneficial effects of dietary probiotics mixture on hemato-immunology and cell apoptosis of *Labeo rohita* fingerlings reared at higher water temperatures. *PLOS ONE*, 9(**6**), 1-9. DOI: 10.1371/journal.pone.0100929

Neissi, A., Rafiee, G., Nematollahi, M. and Safari, O., 2013. The effect of *Pediococcus acidilactici* bacteria used as probiotic supplement on the growth and non-specific immune responses of green terror (*Aequidens rivulatus*). *Fish and Shellfish Immunology,* 35(**6**), 1976-1980. DOI: 10.1016/j.fsi.2013.09.036

Olayinka, A.S. and Afolabi, O.O., 2013. Evaluation of the effects of *Lactobacillus acidophilus* on the haematological parameters of *Clarias gariepinus*. *International Journal of Research in Fisheries and Aquaculture*, 3(**2**), 38-41.

Ongena, M., and Jacquea, Ph., 2007. Bacillus lipopeptides: Versatile weapons for plant disease biocontrol. *Trends in Microbiology*, 16(**3**), 115-125. DOI: 10.1016/j.tim.2007.12.009

Peglia, D.E. and Valentine, W.N., 1976. Studies on the quantitative and qualitative characterization of erythrocyte glutathione peroxidase. *Journal of Laboratory and Clinical Medicine*, 70(**1**), 158-169.

Pereira, L.F., 2014. Growth performance, antioxidant and innate immune responses in European seabass fed probiotic supplemented diet at three rearing temperatures. Portugal: Universidade do Porto. Page53

Pourkazemi, M., 2006. Caspian Sea sturgeon conservation and fisheries: Past, present and future. *Journal of Applied Ichthyology*, 22(**2**), 12-16. DOI: 10.1111/j.1439-0426.2007.00923.x

Salaghi, Z., Imanpoor, M.R. and Taghizadeh, V., 2013. Effect of different levels of Primalac probiotic on growth performance and survival rate of Persian sturgeon (*Acipenser persicus*). *Global Veterinaria*, 11(**2**), 238-242. DOI: 10.5829/idosi.gv.2013.11.2.7545

Shen, W.Y., Fu, L.L., Li, W.F. and Zhu, Y.R., 2010. Effect of dietary supplementation with *Bacillus subtilis* on the growth, performance, immune response and antioxidant activities of the shrimp (*Litopenaeus vannamei*). *Aquaculture Research*, 41(**11**), 1691-1698. DOI: 10.1111/j.1365-2109.2010.02554.x

Siwicki, A.K. and Anderson, D.P., 1993. Non-specific defense mechanisms assay in fish: II. Potential killing activity of neutrophils and macrophages, lysozyme activity in serum and organs and total immunoglobulin level in serum. Poland: Olsztyn. Pages 105-111

Smitha, S. and Bhat, S.G., 2013. Thermostable Bacteriocin BL8 from *Bacillus licheniformis* isolated from marine sediment. *Journal of Applied Microbiology*, 114(**3**), 688–694. DOI: 10.1111/jam.12097

Stolen, J.S., Fletcher, T.C., Anderson, D.P., Roberson, B.S. and Van Muiswinkel, W.B., 1990. Techniques in fish immunology. New Jersey: SOS Publications. Pages 101-103

Subramanian, S., MacKinnon, Sh.L. and Ross, N.W., 2007. A comparative study on innate immune parameters in the epidermal mucus of various fish species. *Comparative Biochemistry and Physiology*, 148(**2**), 256-263. DOI: 10.1016/j.cbpb.2007.06.003

Taati, R., Soltani, M., Bahmani, M. and Zamini, A.A., 2011. Growth performance, carcass composition immunophysiological indices in juvenile great sturgeon (*Huso huso*) fed on commercial prebiotic, Immunoster. *Iranian Journal of Fisheries Sciences*, 10(**2**), 324-335.

Tovar-Ramírez, D., Mazurais, D., Gatesoupe, J.F., Quazuguel, P., Cahu, C., and Zambonino-Infante, J.L., 2010. Dietary probiotic live yeast modulates antioxidant enzyme activities and gene expression of sea bass (*Dicentrarchus labrax*) larvae. *Aquaculture*, 300(**1-4**), 142-147. DOI: 10.1016/j.aquaculture.2009.12.015

Valeur, N., Engel, P., Carbajal, N., Connolly, E. and Ladefoged, K., 2004. Colonization and immunomodulation by *Lactobacillus reuteri* ATCC 55730 in the human gastrointestinal tract. *Applied and Environmental Microbiology*, 70(**2**), 1176–1181. PMID: 14766603. PMCID: PMC348788