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Effect of dietary A-Max supplementation as a prebiotic on growth performance and hemato- immunological parameters of great sturgeon (*Huso huso* Linnaeus, 1758) juveniles

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Abstract

An 8-week feeding trial was conducted to evaluate the effects of dietary commercial prebiotic A-Max, a mixture of mannan oligosaccharides, fructo oligosaccharides and β glucan, on growth and hemato-immunological parameters of great sturgeon (*Huso huso*) juveniles. After acclimation, fish (initial weight of 28.79±0.85 g) were placed into 12 tanks (30 fish per tank). Fish were fed a basal diet (containing 44% protein and 20% lipid) supplemented with 0 (control), 0.5, 1.0 and 1.5 g kg^{-1} A-Max in a totally randomized design in triplicate groups. At the end of the trial, growth factors and haemato-immunological parameters were assessed. Fish fed 1.5 g kg^{-1} prebiotic mixture displayed higher growth performances and feed efficiency compared to the control group (p>0.05). There were no significant differences in survival rate among all treatment groups (p>0.05). The results showed that RBC, WBC, lymphocyte, neutrophil, haematocrit and total protein were significantly affected by 1.5 g kg^{-1} dietary prebiotic mixture (p<0.05). An elevation of monocyte and haemoglobin (p>0.05) was found in the fish fed diet containing 1.0 g kg^{-1} prebiotic. In addition fish fed the diet with 1.5 g kg^{-1} prebiotic mixture showed a significant increase in lyzosyme activity, respiratory burst activity, serum total immunoglobulin (Ig) and alternative complement activity (ACH50) (p<0.05) compared with those fed the diets supplemented with other levels of prebiotics. The results showed that the addition of 1.5 g kg^{-1} prebiotic mixture to the diet of great sturgeon juveniles improving growth performance, some haematological parameters and immune response and seemed to be an effective immunostimulant.

Keywords: Prebiotic mixture, Growth, Hematology, Immune response, Great sturgeon (*Huso huso*)

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Introduction

Growth enhancement and disease resistance of aquaculture organisms are two of the most important concerns (Li *et al*., 2005b). The use of dietary additives in fish farms is one of the methods commonly used to improve weight gain, feed efficiency, and/or disease resistance in cultured fish. Therefore, several kinds of additives for aquafeed that are used to improve the performance of fish have been studied (Cho and Lee, 2012). Recently, immunostimulants such as prebiotics have shown promise as preventive and environmentally friendly alternatives to antibiotics in aquaculture (Sang *et al*., 2009). Prebiotics are non-digestible food ingredients which beneficially affect the host by selectively stimulating the growth and/or activity of health-promoting bacteria in the intestinal tract (Gibson, 2004). A-Max is a commercial prebiotic mixture of mannan oligosaccharides, fructo oligosaccharides and immunostimulating compound such as β-glucan. The mentioned elements are derived from the cell wall of yeast (*Saccharomyces cerevisiae*). Mannan oligosaccharides (MOS) and fructo-oligosaccharides (FOS) are two frequently used prebiotics with unique chemical structures that may improve the gut health and ecosystem of the host in different ways (Ye *et al*., 2011). β-glucans are polysaccharides extracted from yeast cell walls. Their immunostimulating function is linked to the presence of species receptors at the surface of macrophages and other phagocytic cells of fish (Verlhac Trichet, 2010). Intake of prebiotics could significantly modulate the colonic microbiota by increasing the number of specific bacteria and thus changing the composition of the microbiota (Gibson and Roberfroid, 1995). The introduction of prebiotics in sturgeon nutrition could be an interesting alternative to improve feed efficiency and sturgeon health. Sturgeons are valuable species, which are currently highly endangered (Safarpour Amlashi *et al*., 2011). Sturgeon culture has seen considerable progress in recent years because artificial culture up to marketable size is important to reduce pressure on natural populations of sturgeon in the Caspian Sea (Pourkazemi, 1997). Great sturgeon, *Huso huso*, is an important aquaculture species in Russia, Eastern Europe, Japan and Iran. This species is good in aquaculture activities (Mohseni *et al*., 2006). Bans and restrictions on antibiotics as feed additives in fish culture in many countries have resulted in the increase in studies on alternative dietary supplements such as probiotics and prebiotics to enhance the health and production of cultured fish (Hoseinifar *et al*., 2011a). However, despite recent advances on the administration of prebiotics on other species, the information on the efficacy of dietary prebiotic in the culture of sturgeon fish has been limited (Akrami *et al*., 2009; Hoseinifar *et al*., 2011a; Ta'ati *et al*., 2011; Razeghi Mansour *et al*., 2012; Akrami *et al*., 2013). Hence, this study aimed to assess the effect of a prebiotic mixture on the growth performance and haemato-immunological parameters of great sturgeon (*H. huso*) juvenile.

Materials and methods

Prebiotic mixture (*A-Max*)

A-Max is a commercial prebiotic mixture of mannan oligosaccharides (14.62%), fructo oligosaccharides (8.06%) and immunostimulating compound such as β-glucan (15.58%). The mentioned elements are derived from the cell wall of yeast (*S. cerevisiae*).

Diet preparation

To prepare the diets, a commercial pelleted diet (containing 44% protein, 20% lipid, 7% ash and 22.71 MJ kg^{-1} GE) was crushed, mixed with the appropriate prebiotic mixture (A-Max) concentration and water, and made again into the pellets, which were allowed to dry for 18 h at 45 ^{0}C by air circulation and stored at 4 ^{0}C until use. The control diet was prepared by adding only water (Cerezuela *et al*., 2008; Akrami *et al*., 2013). The dietary prebiotic mixture (A-Max) was supplemented at levels of 0 (control), 0.5, 1.0 and 1.5 g kg^{-1} dry food for the four experimental groups. The control group received no prebiotic mixture supplement. The approximate chemical composition of formulated diet was determined according to standard methodology (AOAC, 2005).

Feeding and culture system

Great sturgeon Juveniles were obtained from Shahid Marjani Sturgeon Hatchery Center (Gorgan, Iran) and stocked in the experimental fiberglass tanks (2 m × 2 m × 0.5 m) for 2 weeks before the beginning of the experimental regime, in order to condition the fish to the laboratory system and handling procedures, and then, 360 fish at a mean weight of 28.79±0.85 g were randomly allocated to 12 tanks, with 30 fish in each tank, and three replicates per experimental group. The tanks were connected to a continuous circulating system. Continuous aeration was provided to each tank through air stones connected to a central air compressor. During the experimental period, water temperature, dissolved oxygen and pH were 25.5±1.5 ^{0}C, 4.86±0.28 mg L^{-1} and 7.9±0.2, respectively. During the trial, the fish were hand-fed at a rate of 2-5% of the body weight per day (Razeghi Mansour *et al*., 2012), spread across 3 feeding times (08:00, 14:00 and 20:00 h). The daily weight of feed consumed by the fish in each tank was recorded at the end of each day. Dead fish were recorded, and if a fish died during the day it was assumed that it had not consumed feed that day. The feeding trial was carried out for 8 weeks.

Growth performance

In order to analyze the growth indices all of fish from each tank were weighed every 2 weeks, at least 12 h after the last feeding. The fish were weighed by a digital scale (to the nearest 0.01 g) after they had been anesthetized. Based on the results of the biometry, the daily ration of the fish in the supplemented groups and in the control was determined. At the end of the feeding trial, weight gain (WG%), specific growth rate (SGR), feed conversion ratio (FCR) and survival rate were

calculated according to the following formulae:

Weight gain (WG g) = final weight of fish- initial weight of fish (Tacon, 1990).

Specific growth rate (SGR %/day) = 100× [ln final weight of fish- ln initial weight of fish)]/days of feeding (Hevroy *et al*., 2005).

Feed conversion ratio (FCR) = dry feed fed (g)/ wet weight gain (g) (Hevroy *et al*., 2005).

Survival rate % = 100× [initial number of fish - final number of fish)]/ initial number of fish (Ai *et al*., 2006).

Blood sample collection

At the end of the experiment, 6 fish were sampled randomly from each tank and were anaesthetized with clove solution and about 2 mL of blood was drawn from the caudal vein, using a non-heparinized syringe, after they were starved for 24 h. Then, blood samples were introduced to both heparinized and non-heparinized tubes in order to perform haematological and immunological studies, respectively. For serum isolation, blood samples into non-heparinized tubes were centrifuged at 3000 rpm for 10 min (using a Heraeus Labofuge 400) and the sera were removed with a disposable transfer pipette. The latter was stored in the freezer at -20 °C for analysis of total protein, glucose and Immunological parameters test (Ibrahem *et al*., 2010).

Hemato-biochemical assays

In order to study the haematological parameters, the blood samples were suspended in heparinized tube in order to blood cell investigations. The erythrocyte (RBC) and leukocyte (WBC) counts were determined using a Neubaeur haemocytometer (Blaxhall and Daisley, 1973). Hemoglobin levels (Hb) were obtained by the cyano-methemoglobin spectrophotometry method (Dorafshan *et al*., 2008). Haematocrit was measured using the standard microhematocrit method and reported as percentages. To estimate the differential leukocyte counts (lymphocytes, monocyte and neutrophils) blood smears were prepared, air-dried, fixed in methanol, and stained using May–Giemsa solution (Blaxhall and Daisley, 1973). Total protein and glucose levels were determined by the Biuret and glucose oxidase methods, respectively (Asadi *et al*., 2009).

Immunological assays

Lysozyme activity

Lysozyme level was determined by turbidometric assay according to the method of Ellis (1990). With slight modifications. Aliquots (1.75 mL^{-1}) of *Micrococcus lysodeikticus* suspension (Sigma) (0.375 mg mL^{-1}, 0.05 M PBS, pH 6.2) were mixed with 250 μL^{-1} of each sample and the optical density was measured after 15 and 180 s by spectrophotometer (Biophotometer Eppendorf) at 670 nm. PBS was used as the blank and results were expressed in amounts of lysozyme (µg) per 1 mg of sample calibrated using a standard

curve determined with hens egg white lysozyme (Sigma) in sterile sodium phosphate buffer.

Respiratory burst activity

The generation of intracellular superoxide radicals by sole phagocytes was determined by the reduction of nitro-blue tetra-zolium (NBT) according to the technique described by Secombes (1990) and Boesen *et al*. (2001). Phagocyte monolayers were washed with L^{-15} medium and HBSS (Hank's Balanced Salt Solution) to remove any trace of antibiotics. Then, 100 µL NBT, dissolved at 1mg mL^{-1} in HBSS, were added to the wells and the phagocytes incubated at 22 ºC for 30 min. After incubation, the medium was removed and the culture was washed twice with isotonic PBS, fixed with 100 µL/well of 100% methanol for 3 min, and washed twice with 70% methanol; then, the cells were air dried. Formazan was solubilized in 120 µL of KOH (2 M) plus 120 µL of dimethyl sulfoxide (DMSO) and the absorbance was read spectrophotometrically (Hitachi) at 620 nm using KOH/DMSO as a blank.

Serum total immunoglobulin (*Ig*)

Serum total immunoglobulin (Ig) levels were determined according to the method described by Siwicki and Anderson (1993). Briefly, serum total protein content was measured using a microprotein determination method (C-690; Sigma), prior to and after precipitating down the immunoglobulin molecules, using a 12% solution of polyethylene glycol (Sigma). The difference in protein content represents the Ig content.

Alternative complement activity (*ACH50*)

Alternative complement activity was assayed according to the procedure of Yano (1992). By using rabbit red blood cells (RaRBC). The volume of serum yielding 50% haemolysis was determined and used to calculate the complement activity of the sample (value of ACH50 is in units per mL).

Statistical analysis

The normality and homogeneity of data were explored by examining the residual plots. The data were subjected to one-way analysis of variance (ANOVA), and if significant ($p<0.05$) differences were found, Duncan's multiple range test was used to rank the groups using SPSS (version15).

Results

The effects of the different levels of dietary prebiotic mixture (A-Max, from USA)) on the growth performance and feed utilization of great sturgeon juvenile are shown in Table 1. At the end of the trial, there were no significant differences in growth and feeding parameters such as weight gain, SGR and FCR between juveniles fed control and prebiotic mixture supplementation diets ($p>0.05$). No mortality was observed during the experiment ($p>0.05$).

Table 1: Growth performance and feed utilization of great sturgeon juvenile fed with diets containing different levels of prebiotic (A-Max) for 60 days.

Levels of prebiotic (g kg^{-1}) / Parameters	Control	0.5	1	1.5
Weight gain %	612.7±41.8	628.4±64.9	637.23±56.6	644.2±42.4
SGR (%/day)	3.41±0.11	3.47±0.23	3.54±0.19	3.57±0.16
FCR	0.86±0.04	0.85±0.07	0.83±0.05	0.81±0.02

Data expressed as mean ± SD, (n=30). Values in the same row sharing the same superscript letter are not significantly different (p>0.05).

The effects of the different levels of dietary prebiotic mixture (A-Max) on the innate immune responses of great sturgeon juvenile are shown in Figs. 1–4. All innate immune responses measured (i.e. lysozyme activity, respiratory burst activity, total immunoglobulin and alternative complement activity) were significantly higher (p<0.05) in fish fed the diet with 1.5 g kg^{-1} prebiotic compared to the control group. The lysozyme activity (66.3±4.8 µg mL^{-1}) and respiratory burst activity (1768±87.9 OD620) of serum were significantly affected by 1.5 g kg^{-1} dietary prebiotic mixture (p<0.05). There were no significant difference between the 0.5 and 1 g kg^{-1} prebiotic mixture group and the control group (p>0.05) (Figs. 1 and 2). Significant differences in total immunoglobulin and alternative complement activity (ACH50) levels were observed between the treatments (p<0.05). Fish fed 1.5 g kg^{-1} prebiotic mixture played significantly elevated total immunoglobulin (4.8±0.7mg mL^{-1}) and ACH50 (85.3±12.6 U mL^{-1}) compared to the control group (p<0.05). There were no significant difference between the 0.5 g kg^{-1} prebiotic mixture and the control group (p>0.05) (Figs. 3 and 4).

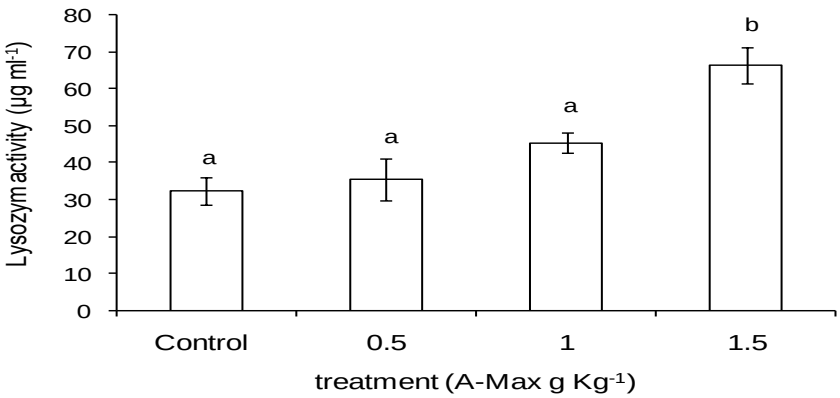

Figure 1: Serum lysozyme activity of great sturgeon juvenile fed with diets containing different levels of prebiotic (A-Max) for 60 days. Data represent the mean±SD. Bars assigned with different superscripts are significantly different (p<0.05).

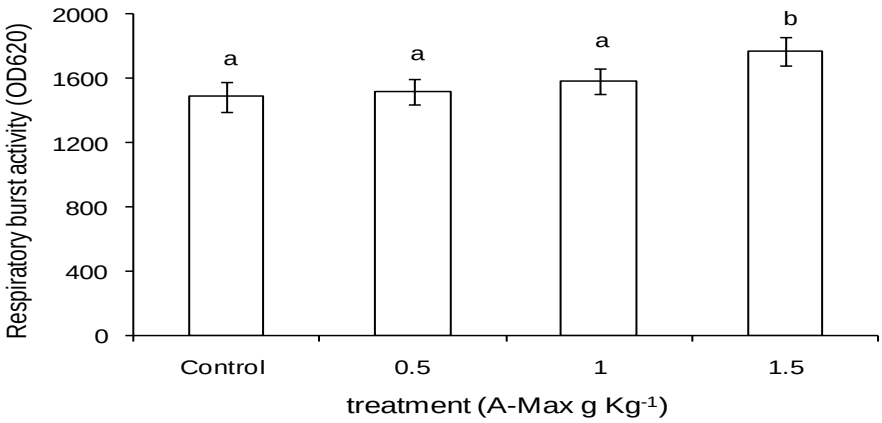

Figure 2: Respiratory burst activity (OD620) of great sturgeon juvenile fed with diets containing different levels of prebiotic (A-Max) for 60 days. Data represent the mean±SD. Bars assigned with different superscripts are significantly different (*p*<0.05).

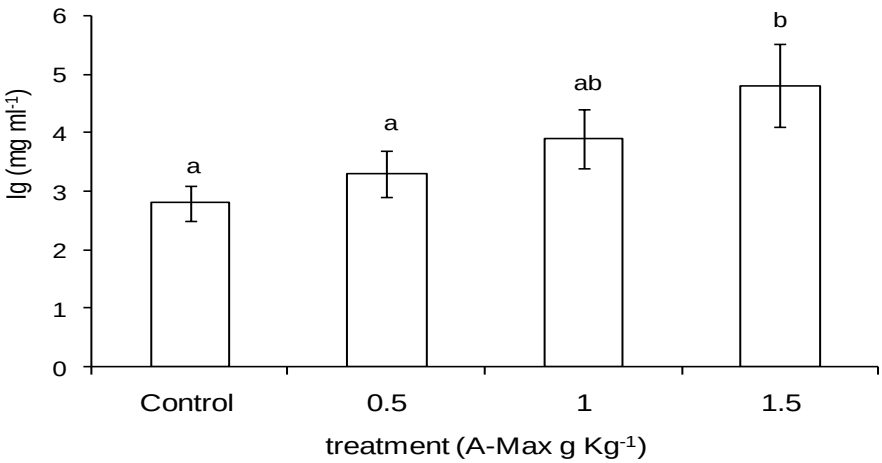

Figure 3: Serum total immunoglobulin (Ig) levels of great sturgeon juvenile fed with diets containing different levels of prebiotic (A-Max) for 60 days. Data represent the mean±SD. Bars assigned with different superscripts are significantly different (*p*<0.05).

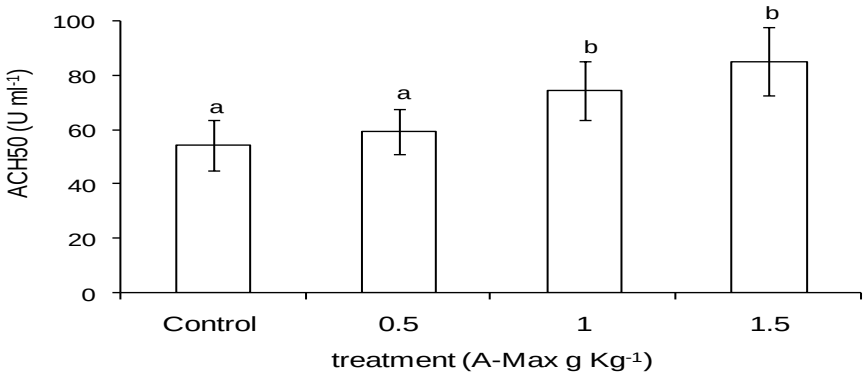

Figure 4: Serum alternative complement activity (ACH50) of great sturgeon juvenile fed with diets containing different levels of prebiotic (A-Max) for 60 days. Data represent the mean± SD. Bars assigned with different superscripts are significantly different (*p*<0.05).

Hematological and biochemical parameters of great sturgeon juvenile fed on different levels of dietary prebiotic mixture (A-Max) are shown in table 2. The number of erythrocyte (RBC), leucocytes (WBC),

lymphocytes, neutrophils and hematocrit were significantly ($p<0.05$) higher in the groups that were fed prebiotic mixture diet at 1.5 g kg^{-1} feed (Table 2). An elevation of hemoglobin level and monocyte count was found following feeding with 1.5 g kg^{-1} prebiotic diet although they were not significantly different ($p>0.05$). Prebiotic mixture added diets affected the blood glucose level, being relatively lower in all treated groups compared with the control (Table 2). The lowest glucose content and highest protein content were found in the fish fed the diet with 1.5 g kg^{-1} prebiotic mixture compared to the control group ($p<0.05$).

Table 2: Hematological and blood serum biochemical parameters of great sturgeon juvenile fed with prebiotic A-Max added diet at different levels for 60 days.

Levels of prebiotic (g kg^{-1}) / Parameters	Control	0.5	1	1.5
RBC (10^6 mL^{-1})	0.8 ± 0.07^c	0.82 ± 0.13^c	1.04 ± 0.1^b	1.56 ± 0.02^a
WBC (10^3 mL^{-1})	18.6 ± 0.95^c	19.2 ± 0.95^c	20.83 ± 0.89^b	22.76 ± 0.21^a
Lymphocytes (%)	74.33 ± 3.78^b	71.51 ± 2.15^b	73.66 ± 1.52^b	79.47 ± 1.94^a
Monocytes (%)	3.9±0.14	3.92±0.17	4.12±0.15	4.18±0.12
Neutrophils (%)	15.32 ± 1.21^b	16.47 ± 1.72^{ab}	17.35 ± 1.52^{ab}	19.68 ± 0.57^a
Hemoglobin (g dL^{-1})	8.06±0.08	8.03 ± 0.07	9.51 ± 0.51	9.91 ± 0.26
Hematocrit (%)	26.16 ± 1.6^b	26.31 ± 3.21^b	30.16 ± 4.1^b	35.76 ± 0.81^a
Glucose (mg $^{-1}$)	76 ± 10.14^a	70 ± 8.5^{ab}	61.66 ± 2^{bc}	56 ± 1^c
Total protein (mg $^{-1}$)	1.8 ± 0.4^a	1.8 ± 0.3^a	2.1 ± 0.5^b	2.3 ± 0.4^b

Data expressed as mean±SD, (n=6). Data assigned with different superscripts indicate significant differences ($p<0.05$).

Discussion

To our knowledge, this is the first study to investigate the effects of A-Max, a mixture of prebiotics on great sturgeon (*H. huso*) juvenile. The result of present study showed that there were no significant differences in growth related parameters such as weight gain, SGR and FCR between great sturgeon juveniles fed control and prebiotic supplemented diets ($p>0.05$). Similar to our results, several other studies have revealed that growth parameters have remained unaffected with prebiotic applications in fish (Pryor *et al*., 2003; Genç *et al*., 2006; Genç *et al*., 2007; Welker *et al*., 2007; Sado *et al*., 2008; Akrami *et al*., 2010; Dimitroglou *et al*., 2010; Peterson *et al*., 2010; Ai *et al*., 2011; Hoseinifar *et al*., 2011b; Peterson *et al*., 2012; Razeghi Mansour *et al*., 2012). The absence of positive significant effects on growth may be attributed to the inability of intestinal microbiota to ferment excessive prebiotic levels and the subsequent accumulation of indigestible material in the intestine which may cause irritation

to the gut (Hoseinifar *et al*., 2011b; Soleimani *et al*., 2012). Also Ye *et al*. (2011) explained that considerable variation in growth, feed utilization and health benefits with the dietary use of prebiotics and/or probiotics is likely dependent on fish species, feeding duration, fish nutritional/physiological status, ambient culturing conditions and supplementation level as well as the type of pre- and probiotics. Growth enhancement as a result of prebiotic administration has been reported in several previous studies on a variety of fish species fed dietary prebiotics (Li and Gatlin, 2004; Li *et al*., 2005a; Mahious *et al*., 2006; Staykov *et al*., 2007; Torrecillas *et al*., 2007; Yilmaz *et al*., 2007; Grisdale-Helland *et al*., 2008; Gultepe *et al*., 2010; Ye *et al*., 2011; Gultepe *et al*., 2012; Soleimani *et al*., 2012; Akrami *et al*., 2013). Unlike this study, Akrami *et al*. (2015) found that there was a significant improvement in the weight gain and feed conversion ratio in rainbow trout treated with 1.5 g kg^{-1} A-Max.

At the end of the feeding trial, haematological parameters such as erythrocyte (RBC), leucococytes (WBC), lymphocytes, neutrophils and haematocrit were improved by the inclusion of 1.5 g kg^{-1} dietary prebiotic. Similar to these results, Andrews *et al*. (2009) observed a significant improvement in WBC, RBC and Hb, in rohu (*Labeo rohita*) fed on the MOS supplemented diet in comparison with those fed on the control diet. On the contrary, Welker *et al*. (2007) reported that RBC, WBC, Hct, Hb and plasma protein levels were not affected in Channel catfish (*Ictalurus punctatus*) fed 0.2% mannan oligosaccharide. Sado *et al*. (2008) and Gultepe *et al*. (2012) showed that dietary MOS had no significant effect on hematological parameters of Nile tilapia (*Orechromis niloticus*) and Gilthead Seabream (*Sparus auratus*) respectively. Also Hoseinifar *et al*. (2011b) and Razeghi Mansour *et al*. (2012) explained that supplementation with fructooloigosaccharide and mannan oligosaccharide had no effects on hematological parameters of giant sturgeon (*H. huso*) juvenile. Stress responses by the fish as a result of daily feeding on b-glucan may increase RBC, WBC, Hct and Hb of the blood. Ebrahimi *et al*. (2012) also reported increased WBC counts in common carp (*Cyprinus carpio*) after feeding on Immunogen prebiotic which is a mixture of mannan oligosaccharide and β-glucans. The observed increases in the leucocyte and total protein appear to be signs of enhanced health status of the prebiotic-fed fish.

In the present study, the lowest glucose content and highest protein content were found in the fish fed diet 1.5 g kg^{-1} prebiotic mixture compared to the control group ($p<0.05$). Andrews *et al*. (2009) showed a significant improvement in serum protein in *L. rohita* fed on the MOS supplemented diet in comparison with those fed on the control diet. On the contrary, Welker *et al*. (2007) and Sado *et al*. (2008) reported that plasma protein levels was not affected in Channel catfish and tilapia fed mannan oligosaccharide (MOS), respectively. Also, Hoseinifar

et al. (2011a) observed that glucose and total protein levels were not affected in beluga fed FOS. Subsequently, Akrami *et al*. (2015) studied the effects of dietary intake of prebiotic mixture (A-Max) in rainbow trout, and they observed that this supplement cannot significantly affect serum total protein, albumin, and glucose levels.

Stimulation of the immune response of fish through dietary supplements is of high interest for commercial aquaculture (Soleimani *et al*., 2012). The innate immune system is very important in this regard because aquatic animals are continually vulnerable to numerous opportunistic pathogens and this part of immune response provides the first line of defense for the host (Magnadóttir, 2006). The result of the present research showed that lysozyme activity increased significantly in the group treated with 1.5 g kg^{-1} prebiotic ($p<0.05$). Similar to these results, Soleimani *et al*. (2012) observed a significant improvement in serum lysozyme activity and serum alternative complement activity (ACH50) in Caspian roach (*Rutilus rutilus*) fry fed on the FOS-supplemented diet in comparison with those fed on the control diet. In another research, serum lysozyme activity enhanced significantly in stellate sturgeon (*A. stellatus*) juveniles fed on the diet supplemented with 1% fructooligosaccharide compared with other groups (Akrami *et al*., 2013). Similarly, application of FOS as prebiotic was found to enhance the lysozyme activity of Nile tilapia (*O. niloticus*) (He *et al*., 2003), Red drum (*Sciaenops ocellatus*) (Zhou *et al*., 2010) and Japanese flounder (*Paralichthys olivaceus*) (Ye *et al*., 2011). In contrast to these results, no significant effects were observed on serum lysozyme activity of Atlantic salmon (*Salmo salar*) and Siberian sturgeon (*Acipenser baerii*) fed on FOS and arabinoxylan-oligosaccharides compared with the control diet, respectively (Grisdale-Helland *et al*., 2008; Geraylou *et al*., 2012). This contradictory result may be attributable to the low dosage (Soleimani *et al*., 2012), degree of polymerization (Geraylou *et al*., 2012), different duration of prebiotic administration, life stage and/or different fish species (Geraylou *et al*., 2012; Soleimani *et al*., 2012).

In the current study, respiratory burst activity, total immunoglobulin and alternative complement activity increased significantly in the great sturgeon juveniles with 1.5 g kg^{-1} prebiotic compared with control group ($p<0.05$). Similarly, Geraylou *et al*. (2012) and Akrami *et al*. (2013) showed a significant improvement in alternative complement activity (ACH50) in Siberian sturgeon and stellate sturgeon fed on the arabinoxylan-oligosaccharides and MOS supplemented diet in comparison with those fed on the control diet, respectively. Additionally, Soleimani *et al*. (2012) observed a significant improvement in serum total immunoglobulin (Ig) levels in Caspian roach (*R. rutilus*) fry fed on the FOS-supplemented diet. On the contrary, no significant differences in respiratory

burst activity were observed for the Siberian sturgeon (*A. baerii*) fed arabinoxylan-oligosaccharides (Geraylou *et al*., 2012) and stellate sturgeon (*A. stellatus*) fed with FOS (Akrami *et al*., 2013).

In conclusion, the result indicated that prebiotic mixture (A-Max) at the level of 1.5 g kg^{-1} in the diet of great sturgeon juvenile improved growth performance, immune response and some blood parameters of great sturgeon juvenile and it is appropriate for supplementation in the diet of cultured great sturgeon juveniles.

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