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Original Article

Influence of dietary prebiotic mixture (*α-mune*) on growth performance, haematology and innate immunity of Beluga sturgeon (*Huso huso*) juvenile

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Abstract: The present study investigated the effects of prebiotic (α-mune), a mixture of mannan oligosaccharide, β-1,3 and β-1,6 glucan, on the growth performance, haematology and innate immunity of beluga sturgeon (*Huso huso*) juvenile. Fish (46±3 g) were allocated into 12 tanks (15 fish per tank) and triplicate groups were fed a control diet or diets containing 1.5, 3.0 and 4.5 g kg^{-1} prebiotic for 46 days. Fish fed 1.5 g kg^{-1} prebiotic displayed significantly higher final weight, specific growth rate and feed conversion ratio. WBC, RBC, MCV, MCH, haemoglobin, haematocrit and lymphocyte levels were also significantly higher in the fish fed 1.5 g kg^{-1} prebiotic. Furthermore, the highest haematocrit content and lymphocyte level were found in the fish fed a diet containing 1.5 g kg^{-1} prebiotic. Alternative complement activity (ACH50), lysozyme activity and Ig concentration were significantly higher in the fed 1.5 g kg^{-1} prebiotic. These results indicate that α-mune can be considered as a beneficial dietary supplement for improving the growth performance, haematological and immunological parameters of beluga sturgeon juvenile.

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Introduction

Beluga sturgeon, *Huso huso*, has been classified as a critically endangered species in the Caspian Sea (Mohseni et al., 2006). Along with declining the natural populations of this species, its farming has been developed across the world (Abdolhay and Tahori, 2006; Mohseni et al., 2006). This developmental trend has been attributed to excellent characters of this species such as fast growth, easy adaptation to farm condition, and specifically, its expensive caviar (Mohseni et al., 2006). Since, little information is available about the nutritional requirements of *H. huso* in aquaculture (Hoseinifar et al., 2011b), hence, the introduction of prebiotics in sturgeon nutrition could be an interesting alternative to improve their feed efficiency and health.

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). In this regard, the beneficial effects of dietary mannan oligosaccharides and β-glucan on fish and crustacean species have been reported. These prebiotics can positively influence the haematological parameters and non-specific immune responses (Staykov et al., 2007; Andrews et al., 2009; Ta'ati et al., 2011; Ye et al., 2011; Soleimani et al., 2012) as well as growth performance (Mahious et al., 2006; Torrecillas et al., 2007; Staykov et al., 2007; Yilmaz et al., 2007; Grisdale-Helland et al., 2008; Piaget et al., 2007; Gultepe et al., 2010; Ye et al., 2011; Gultepe et al., 2012) of fish species.

Since, analysis of growth performance, haematological parameters and innate immunity are proper indicators for evaluating the conditions of aquatic animals (Bahmani et al., 2001). Hence, the

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present study was conducted to evaluate the effects of different levels of the prebiotic mixture (α-mune, a mixture of mannan oligosaccharide, β-1,3 and β-1,6 glucan) on growth performance, haematology and innate immunity of the juvenile beluga sturgeon.

Materials and Methods

Diet preparation: To prepare the experimental diets, a commercial pellet diet (Skretting) supplemented with 0 (control), 1.5, 3.0 and 4.5 g kg^{-1} prebiotic *α-mune* (Alfarma, Belgium) in triplicate group. For this purpose, the commercial pellet was crushed, mixed with the appropriate prebiotic concentrations and water, and pelleted; then were allowed to be dried for 18 hrs at 45°C by air circulation and stored at 4°C. The control diet was prepared by adding only water (Cerezuela et al., 2008) (Table 1).

Feeding trial: Juveniles were obtained from the Shahid Marjani Sturgeon Hatchery Center (Gorgan, Golstan Province, Iran). Prior to experiment, fish were acclimated to experimental conditions for one week and fed by commercial diet without prebiotic to acclimatize for experimental diet. At the beginning of the experiment, the juveniles with an initial average weight of 46±0.8 g were randomly distributed into 12 fibreglass tanks (2×2×0.5 m) at a stocking density of 15 fish per tank. Water temperature, dissolved oxygen, pH and salinity were monitored daily and maintained at 26.12±0.58°C, 6.55±0.49 mg L^{-1}, 8.04±0.08 and 1.26±0.28, respectively. During the trial, fish were hand-fed 1-3% of the body weight 3 times a day at 07:00, 13:00 and 19:00 h for 46 days.

Growth performance: Total fish weight in each tank was measured every 15 days to adjust the feeding rate and estimate growth performance. At the end of the experiment, the fish were fasted for 24 hrs before harvest and weighing. The total numbers were counted and the mean body weights of fish were measured. Then weight gain (WG), specific growth rate (SGR), feed conversion ratio (FCR) and condition factor (CF) of each tank were calculated using the following equations:

Weight gain (WG, g) = final weight (g) - initial weight (g)

Specific growth rate (SGR% / day) = 100× [ln final weight - ln initial weight)] / days of feeding

Feed conversion ratio (FCR) = dry feed intake (g) / wet weight gain (g)

Condition factor (CF) = final weight (g) / (fork length (cm))^3 ×100

Survival rate (%) = 100× [initial number of fish - final number of fish)]/ initial number of fish.

Table 1. Proximate composition of the commercial diet (skretting).

Ingredients	% dry weight
Crude protein	50
Crude lipid	20
Ash	7.5
Fiber	7
NFE [1]	15.5
Gross energy (MJ/kg) [2]	22.33

1 Nitrogen free extract (NFE) = 100 - (% crude protein + % crude lipid +% ash +% fiber)

2 Gross energy (GE) (MJ/kg) = (% crude protein × 23.6 + % crude lipid × 39.5 + % NFE × 17)

Chemical analyses: Chemical analysis of formulated diet was performed according to AOAC (2005) (Table 1). Crude protein content (N×6.25) using a Kjeldahl system (Buchi, Switzerland), crude lipid content by a soxhlet system (Buchi, Switzerland), ash content by weighting after incinerating at 550ºC for 6 hrs in a hotspot furnace (Gallenkamp, England), and crude fiber content by an automatic analyzer (Fibertec, Sweden) were measured.

Blood sample collection and haematological parameters: At the end of the experiment, 3 fish were randomly sampled from each tank. After anesthetizing with clove solution, about 2 ml of blood was sampled from the caudal vein, using a non-heparinized syringe. Then, the blood samples were introduced to both heparinized (EDTA) and non-heparinized tubes to perform haematological and immunological analysis, respectively. Serum samples were attained after centrifugation (4,500×g for 10 min) and stored at -20°C until analysis for Immunological studies.

Red blood cells (RBC) and white blood cells

(WBC) were counted using a Neubaur haemocytometer according to Martins et al. (2004). Haemoglobin (Hb) according to Collier (1944); haematocrit (Hct) according to Goldenfarb et al. (1971); mean corpuscular volume (MCV) and mean corpuscular haemoglobin (MCH) according to Wintrobe (1934), and differential white blood cell counts based on Klontz (1994) were other measured haematological parameters.

Immunological assays:

Alternative complement activity (ACH50): The serum complement activity was determined by assaying the alternative complement activity (ACH50) (Yano et al., 1988). Rabbit blood was mixed with an equal volume of Alsevers's solution and stored at 4°C. Subsequently, the cells were centrifuged at 400 g for 5 min; the pellet of RaRBC was washed twice in 10 mM ethylene glycolbis tetraacetic acid (EGTA)-Mg-gelatin veronol buffer (GVB) and suspended in the same buffer at a concentration of 2×10^8 cells ml^{-1}. Alternative complement pathway activity was assayed according to Yano et al. (1988). Briefly, 0.5 ml of serially 10-fold diluted carp serum in EGTA–Mg–GVB was placed in a set of test tubes and 0.2 ml of sheep red blood cells suspension (2×10^6 cells ml^{-1}) was added. This mixture was incubated at 15°C for 90 min. Addition of 2.8 ml of 10 mM EDTA-GVB buffer stopped the hemolytic reaction. After centrifugation, the value y (percent haemolysis /100) was calculated from the optical density at 414 nm of the supernatant. The value y/ (1-y) and the reciprocal of the serum dilution were plotted on log-log graph paper and the ACH50 (U ml^{-1}), the reciprocal dilution giving 50% haemolysis [y (1-y) =1], was read from the graph (Sakai et al., 2001).

Lysozyme activity: Lysozyme level was determined by turbidity 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 spectro-photometer (Biophotometer Eppendorf) at 670 nm. PBS was used as a blank and results were expressed according to amounts of lysozyme (μg) per 1 mg of sample calibrated using the standard curve determined with egg white lysozyme (Sigma) in sterile sodium phosphate buffer.

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

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 (Duncan, 1995) was used to rank the groups using the SPSS (Version 15).

Results

No mortality was recorded throughout the experiment. Effects of different dietary prebiotic *α-mune* levels on the growth performance and feed utilization are shown in Table 2. At the end of the experiment, fish fed 1.5 g kg^{-1} prebiotic displayed significantly improved growth performance and feed utilization, including WG, SGR and FCR than other treatments ($P<0.05$). Also, a non-significant elevation of CF was found in the fish fed diet 1.5 g kg^{-1} prebiotic ($P>0.05$).

Effects of different levels of dietary prebiotic *α-mune* on the haematological parameters are presented in Table 3. WBC, RBC, MCV, MCH, haemoglobin, haematocrit and lymphocyte levels were significantly higher in the beluga fed 1.5 g kg^{-1} prebiotic compared to other groups ($P<0.05$). The highest monocyte level was observed in beluga juveniles fed control diet ($P>0.05$).

Immunological parameters of beluga sturgeon juvenile fed different levels of the dietary prebiotic *α-mune* are shown in Figures 1-3. After the 46 days

Table 2. Growth performance of beluga juvenile fed the diets containing various prebiotic *α-mune* levels for 46 days.

Levels of prebiotic / Parameters	Control	1.5 g kg $^{-1}$	3.0 g kg $^{-1}$	4.5 g kg $^{-1}$
WG (g)	161.0± 4.9^{b}	173.24± 4.5^{a}	154.78± 3.7^{b}	158.17± 1.6^{b}
SGR (%/day)	3.27± 0.05^{b}	3.39± 0.05^{a}	3.19± 0.04^{b}	3.23± 0.00^{b}
FCR	1.37± 0.01^{ab}	1.27± 0.11^{b}	1.41± 0.02^{a}	1.38± 0.05^{ab}
CF (%)	0.48± 0.02^{a}	0.52± 0.05^{a}	0.47± 0.00^{a}	0.48± 0.02^{a}
Survival (%)	100	100	100	100

Data assigned with different superscripts indicate significant differences (P<0.05).

Table 3. Haematological parameters of beluga juvenile fed the diets containing various prebiotic *α-mune* levels for 46 days

Levels of prebiotic / Parameters	Control	1.5 g kg $^{-1}$	3.0 g kg $^{-1}$	4.5 g kg $^{-1}$
WBC (per/mm^{3})	6850± 132.28^{b}	7350± 150.00^{a}	6616.7± 246.64^{b}	6600± 200.00^{b}
RBC (per/mm^{6})	0.81± 0.00^{b}	0.86± 0.01^{a}	0.82± 0.00^{b}	0.82± 0.00^{b}
MCV (fl)	227.60± 1.15^{c}	236.74± 2.23^{a}	230.60± 2.34^{bc}	231.35± 1.07^{b}
MCH (pg)	75.97± 0.94^{b}	81.33± 1.01^{a}	77.13± 1.01^{b}	77.38± 0.57^{b}
Haemoglobin (g/dl)	6.20± 0.10^{b}	6.83± 0.05^{a}	6.36± 0.15^{b}	6.40± 0.10^{b}
Haematocrit (%)	18.60± 0.30^{b}	19.66± 0.25^{a}	19.03± 0.40^{b}	19.13± 0.25^{ab}
Monocyte (%)	4.33± 1.15^{a}	3.00± 1.00^{a}	4.00± 1.00^{a}	3.66± 0.57^{a}
Lymphocyte (%)	74.33± 3.78^{ab}	79.00± 1.00^{a}	75.33± 2.51^{b}	73.00± 2.00^{c}
Basophil (%)	0.66± 0.57^{a}	0.66± 0.57^{a}	0.66± 0.57^{a}	0.66± 0.57^{a}

Data assigned with different superscripts indicate significant differences (P<0.05).

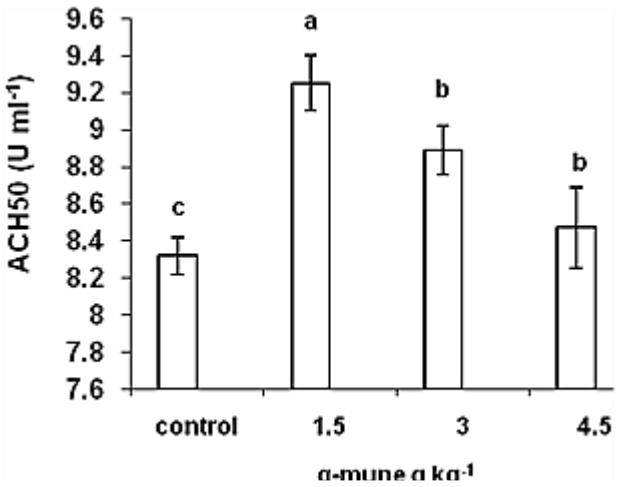

Figure 1. Serum alternative complement activity (ACH50) of beluga juvenile fed the diets containing various prebiotic α-mune levels for 46 days. Data assigned with different superscripts indicate significant differences (P<0.05).

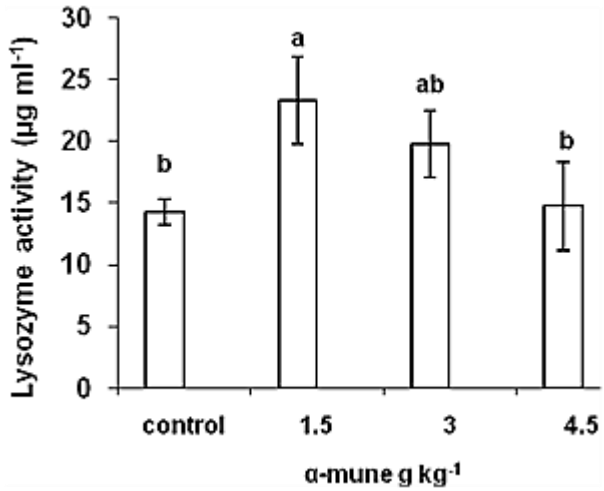

Figure 2. Serum lysozyme activity of beluga juvenile fed the diets containing various prebiotic α-mune levels for 46 days. Data assigned with different superscripts indicate significant differences (P<0.05).

feeding trial, alternative complement activity (ACH50) and Ig concentration were significantly higher in the fish fed 1.5 g kg^{-1} prebiotic than other groups (P<0.05). Furthermore, lysozyme activity was significantly higher in fish fed 1.5 g kg^{-1} prebiotic compared to fish fed control and 4.5 g kg^{-1} prebiotic treatments (P<0.05).

Discussion

During the last decade, the use of dietary compounds with potential prebiotic effects is being considered as a possible tool for improving gut health and growth performance of farmed animals (Ortiz et al., 2012). The present study showed that the highest growth

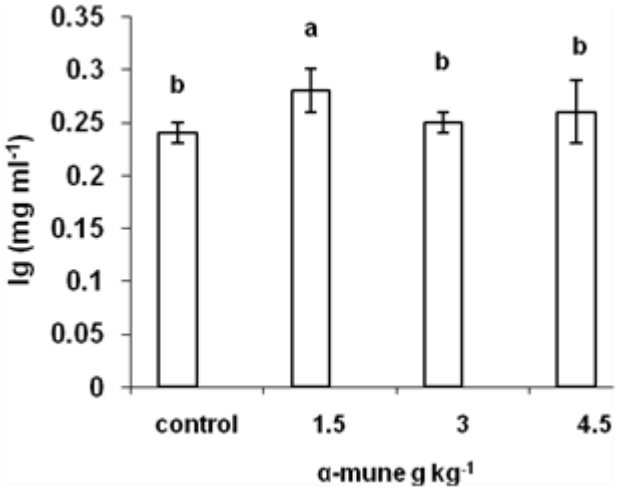

Figure 3. Serum total immunoglobulin (Ig) levels of beluga juvenile fed the diets containing various prebiotic *α-mune* levels for 46 days. Data assigned with different superscripts indicate significant differences (P<0.05).

performance and other measured parameters including WG, SGR and FCR 1.5 g kg^{-1} prebiotic treatment. Mannan oligosaccharide promotes the growth of beneficial lactic acid bacteria in the intestine and these bacteria can inhibit the growth of pathogens by producing bacteriosins (Andrews et al., 2009). Although previous studies have showed that dietary oligofructose and mannan oligosaccharide have no effect on growth performance of beluga (Hoseinifar et al., 2011b; Razeghi Mansour et al., 2012). Stimulation of growth by prebiotic has previously been reported in other aquatic animals such as turbot (*Psetta maxima*) (Mahious et al., 2006), rainbow trout (*Oncorhynchus mykiss*) (Staykov et al., 2007; Yilmaz et al., 2007), sea bass (*Dicentrarchus labrax*) (Torrecillas et al., 2007), Atlantic salmon (*Salmo salar*) (Grisdale-Helland et al., 2008), flounder (*Paralichthys adspersus*) (Piaget et al., 2007), sea bream (*Sparus aurata*) (Gultepe et al., 2010, 2012) and Japanese flounder (*paralichthys olivaceus*) (Ye et al., 2011) due to promoting the synthesis of vitamins and enzymatic activity (Lin et al., 2012). However, there are reports about negative effects of dietary prebiotic on other fish species (Pryor et al., 2003; Genc et al., 2006; Genc et al., 2007; Welker et al., 2007; Sado et al., 2008; Dimitroglou et al., 2010; Akrami et al., 2009; Akrami et al., 2010).

Haematological parameters are indices of fish health as well as the physiological status of an organism (Yousefi et al., 2012). The present study indicated that haematological parameters were significantly higher in the beluga fed 1.5 g kg^{-1} prebiotic. White blood cells are used as indicators of health status in fish because of involving in the regulation of immunologic function (Ballarin et al., 2004). In this study, white blood cells showed enhanced levels in the 1.5 g kg^{-1} prebiotic diet; also, lymphocyte and haematocrit cell counts were higher. Ebrahimi et al. (2012) reported that increase in WBC count might be due to stress as a result of daily feeding on β-glucan. Ahmadifar et al. (2010) and Hoseinifar et al. (2011a) were studied the effects of dietary inulin and fructooligosaccharide on the beluga sturgeon juvenile, respectively, and pointed out that WBC levels, lymphocyte and haematocrit cell counts were significantly higher in the fish fed prebiotic. In addition, Andrews et al. (2009) observed a significant improvement in WBC, RBC and Hb, in *Labeo rohita* fed on diets supplemented with mannan oligosaccharide. In contrast, Razeghi Mansour et al. (2012) reported that different levels of mannan oligosaccharide has no effect on haematological parameters of beluga sturgeon juvenile. Also Similar results have been reported in mannan oligosaccharide fed channel catfish (*Ictalurus punctatus*) (Welker et al., 2007), and Nile tilapia (*Oreochromis niloticus*) (Hisano et al., 2007; Sado et al., 2008). It appears that fluctuations in hematological and biochemical variables may be species specific, inclusion rates of prebiotic, ingredients of diets, rearing period, etc (Ta'ati et al., 2011).

Probiotics and prebiotics stimulate the host immune system (Ye et al., 2011). Stimulation of the immune response of fish through dietary supplements is of high interest for commercial aquaculture (Staykov et al., 2007). The present study showed that dietary prebiotic can modulate the innate immune responses of beluga sturgeon juvenile. Based on the results, beluga sturgeon fed 1.5 g kg^{-1} prebiotic has significantly higher ACH50

and Ig concentration. These data are in agreement with the increased ACH50 and Ig level recorded in roach (*Rutilus caspicus*) fed with the different levels of fructooligosaccharide (FOS) (Soleimani et al., 2012). Serum lysozyme activity was significantly higher in 1.5 g kg^{-1} treatment than that of 4.5 g kg^{-1} and such a result was found by Staykov et al. (2007), Soleimani et al. (2012) and Akrami et al. (2013). The increased serum lysozyme activity indicates that immune system is enhanced in treated fish. In contrast, lysozyme activity was lower in fish fed a MOS diet in Atlantic salmon (Grisdale-Helland et al., 2008). Furthermore, Ye et al. (2011) was evaluated the effects of different levels of dietary FOS, mannan oligosaccharides and *Bacillus clausii* on the Japanese flounder (*Paralichthys olivaceus*) and explained that the FOS, MOS and FOS+MOS dietary supplementations did not alter lysozyme activity. This contradictory result could be referred to the duration of prebiotic administration, the age and type of the treated fish (Ibrahem et al., 2010).

In conclusion, the results of present study showed that dietary administration of *α-mune* as a prebiotic at the level of 1.5 g kg^{-1} can positively influence on growth, haematology and innate immunity of beluga juvenile.

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