Iranian Journal of Fisheries Sciences 20(4) 1165-1178 2021
DOI: 10.22092/ijfs.2021.124567

Research Article

Effects of canola protein hydrolysate (CPH) on growth performance, blood biochemistry, immunity, and gastrointestinal microbiota of beluga (*Huso huso*) juveniles

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Received: December 2020 Accepted: April 2021

Abstract

This study assessed the effects of diets supplemented with canola protein hydrolysate (CPH) on growth performance, blood biochemistry, immunity, and gastrointestinal microbiota of beluga (*Huso huso*) juveniles. CPH concentrations of 0, 300, 400, and 500 mg kg^{-1} diet were denoted as control, CPH_{300}, CPH_{400}, and CPH_{500}, respectively and were added to fish basal diet. 840 beluga juveniles (30±6 g) were assigned into the four dietary treatments and fed for 56 days. The diet supplemented with 500 mg kg^{-1} of CPH significantly decreased food conversion ratio (FCR). Condition factor (CF), specific growth rate (SGR), body weight gain (BWI), standard length (SL), total length (TL), survival rate, and food consumption demonstrated a CPH dose-dependent increase, with highest values in the fish treated with CPH_{500}. Concentrations of blood (serum) cholesterol, triglyceride, and glucose decreased in a CPH-concentration dependent manner as well. Red blood cells (RBC) and white blood cells (WBC) increased and decreased, respectively in all fish treated with CHP, especially in CPH_{500} treatment. Moreover, gastrointestinal population of *Lactobacillus* spp. and total bacteria were significantly enhanced in the treated groups, with the highest loads of total bacterial (2.1×10^6 CFU) and *Lactobacillus* spp. (1.6×10^4 CFU) in CPH_{500} treatment. Taken together, CPH could be introduced as an alternative for fishmeal in beluga diet, although more studies are needed to be conducted to elucidate the underlying mechanisms by which CPH improves fish growth.

Keywords: Bacterial population, Canola, Growth performance, Hydrolyzed protein

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[DOR: 20.1001.1.15622916.2021.20.4.18.3]

Introduction

In aquaculture industry fishmeal is the main standard dietary protein source for many fish species and is mainly produced from wild marine fish. However, given that fishmeal production is ecologically and environmentally unsustainable, it is necessary to find alternative proteins to alleviate economic pressure on aquaculture industry. Plant proteins are the most practical alternatives for fishmeal in aquafeeds (Egerton *et al.*, 2020).

During the last three decades, more than 80% of sturgeon species have been registered in the International Union for Conservation of Nature (IUCN) Red List. Anthropogenic manipulations such as overfishing, environmental pollution, and loss of natural habitats are among the main causes of their population decline worldwide (Asadi *et al.*, 2006; Tashjian *et al.*, 2006; Falahatkar *et al.*, 2015). Hence, the aquaculture production of sturgeon species has attracted attention as a promising approach to recruit their local population through mitigating the fishing pressure and also to meet the market demand (Kalbassi *et al.*, 2013). To develop their aquaculture production, however, it is an urgent need to produce satisfactory diets with lower dependency on marine originated protein sources. That is, it is of high priority to formulate a cost-effective and growth-stimulating diet using terrestrial constituents instead of fishmeal. In this regard enormous constant experimental efforts have been made to incorporate plant-originated nutrients, such as proteins, lipids, and carbohydrates in fish and shellfish diets (Kamalam *et al.*, 2017).

Canola protein hydrolysate (CPH) is used in the diets of other aquatic animals and poultry, but this study first used it in the diet of beluga juveniles. A protein source with high utilization efficiency is important for sustainable development of aquaculture.

In this regard Gui *et al.* (2010) studied the effect of different levels of hydrolyzed protein of flaxseed meal on growth, digestibility, body composition and chemical composition of common carp blood serum and recommended an optimal level of 5%. Utilization efficiency of protein sources, such as fish processing by-products, animal byproducts, and plant protein sources could be improved through enzymolysis process. Hydrolyzed peptides could function as agents with antioxidant, growth stimulant, immunostimulant, antimicrobial properties (Chu and Robinson, 2001; Pasupuleti, 2006; Shobharani and Agrawal, 2009; Wang, 2017). Moreover, hydrolyzed proteins can increase bacterial population (i.e. probiotics) of the intestinal system as well as improve food absorption and body composition of fish (Hevrøy *et al.*, 2005; Gui *et al.*, 2010).

Beluga sturgeon (*Huso huso*) is classified as a critically endangered species in Caspian Sea (Amirkolaie *et al.*, 2012; Hung, 2017). However, some features such as its fast growth, high adaptability to controlled conditions, and its expensive caviar have progressively increased attraction of

[DOR: 20.1001.1.15622916.2021.20.4.18.3]

sturgeon farmers around the world (Abdolhay and Baradaran Tahori, 2006; Mohseni *et al.*, 2008). The use of protein sources for carnivorous fish imposes a high cost on the industry. This issue along with great desire of aquaculturists to use plant sources to reduce food costs lead to the use of plant protein hydrolysis technique for digestion. In addition, their absorption to increase productivity has become very important and practical (Lacki and Duvnjak 1998). In a study (Hevrøy *et al.*, 2005), increasing the hydrolyzed fish protein increased the food uptake in salmon, but the best growth rate was obtained with diets containing moderate amounts of hydrolyzed protein. Nutritionally successful aquaculture production of beluga relies on its nutrient requirements; that is, the profitability of aquaculture production mostly depends on a suitably formulated diet with constituents such as proteins, fats, vitamins, and minerals (Chebanov and Billard, 2001). Therefore, the present research was conducted to assess the effects of canola hydrolyzed peptides on growth performance, blood biochemical and immunological indices, and intestinal bacterial population of beluga sturgeon (*Huso huso*) juveniles.

Materials and methods

Protein hydrolysis

To produce experimental hydrolyzed peptides, defatted canola meal powder was mixed with distilled water (1:10 w/v) and the mixture pH was adjusted at 12 using 0.1 M NaOH (Karimzadeh *et al.*, 2016). The mixture was then heated at 45°C for 30 min and centrifuged at 10000 rpm for 10 min. After the supernatant was collected and 0.1 M HCl was added to adjust the pH at 4 (i.e. isoelectric point), deposited proteins were dissolved in distilled water and the pH was adjusted at 7 using 0.1 M NaOH. The suspension was lyophilized, frozen at -30°C and dried using a freeze dryer. The isolated protein complex was kept in a polyethylene bag and stored at -20°C until further analysis. Then the protein complex was distilled in a reactor placed on a magnetic stirrer and hydrolyzed using Alcalase (Denmark Novozymes Co.) at an enzyme-substrate ratio of 1:20 for 4h. During hydrolysis process, pH was adjusted at 8.0 with 1 M NaOH in a temperature-controlled water bath (50°C). After hydrolysis, the enzyme was inactivated through heating at 90°C for 10 min. The suspension was centrifuged at 8000×g for 30 min and the peptide mixture was then lyophilized at 4°C (Karimzadeh *et al.*, 2016).

Characteristics of hydrolysates

Molecular weight of peptides in the mixture was determined using a high-performance liquid chromatography (HPLC) equipped with a TSK G2000 column. Briefly, the hydrolysate was dissolved in water containing sodium dodecyl sulfate (3g kg^{-1}), centrifuged at 2000g for 10 min, and filtered before injecting into the column. Acetonitrile/water solution (1:1, v/v) containing trifluoroacetic acid (0.1%, v/v) was applied as the mobile phase.

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The absorption was conducted at 225 nm wavelength and a flow rate of 0.5 mL/min. Bovine serum albumin, cytochrome C, and bacteriocin were used as molecular weight standards to define standard curve to relate molecular weight of peptides to elution time (Karimzadeh *et al.*, 2016).

Feeding trial

The growth trial was conducted in summer 2019 for 56 days at Caspian Sea Ecology Research Center, Sari, Iran. A total of 840 beluga juveniles (30g±6) were obtained from a local hatchery center and randomly distributed into twelve 2400L fiberglass tanks (70 fish per each one) and adapted to laboratory condition for two weeks. The tanks were supplied with flow-through water (1.5L min^{-1}). During adaptation period, the fish were fed with a basal diet (i.e., without canola protein hydrolysate; Table 1). For the main dietary experiment, the fish were starved for 48h and then fed thrice daily with the experimental diets containing different levels of hydrolyzed canola proteins (0, 300, 400, 500 mg kg-1 basal diet). Hydrolyzed protein per kg was added to the commercial diet by lubricating the surface of the diet with fish oil (the same amount for all diets). Therefore, according to the number of diets and based on a completely randomized design, four experimental treatments each with three replications were considered.

Table 1: Composition and proximate analysis of the basal diet (as percentage dry weight) for beluga (*Huso huso*) juveniles (100 g^{-1} diet).

Ingredients	Percentage
Fish meal	59.25
Wheat meal	15
Fish oil	6
Soybean oil	6
Starch	1
Mineral mix	3
Vitamin mix	2
Antioxidant	0.25
Antifungi	0.5
Filler	1.74
Total	**100**
Proximate analysis diet%	
Crud protein	44.82
Crud lipid	18.92
Ash	9.53

Each group of fish were fed their respective diet by hand to satisfaction (visual observation of first feed refusal) and feed consumed was recorded (Hosseini *et al.*, 2010). For simplification, hereafter the diets were denoted as CPH_0 (control), CPH_{300}, CPH_{400}, and CPH_{500}, respectively. Feeding trial was carried out under a 12h: 12h light: dark cycle condition, and water temperature, dissolved oxygen, total hardness, and pH were 14±0.3°C, 4-5.1±0.21 mg L^{-1}, 315.2±12.3 mg L^{-1}, and 7.8±0.3, respectively.

Growth performance

At the end of the feeding trial, body weight increase (BWI), specific growth rate (SGR), survival rate, condition factor (CF), and food conversion ratio

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(FCR) of fish were calculated as follows (Jami *et al.*, 2019):

Weight gain (WG%) = 100 × (final body weight (g)- initial body weight (g) / (initial body weight) (g)/ number of days.

Specific growth rate (SGR%)=100×(final weight of fish (g) - initial weight of fish (g) /days of feeding

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

Feed conversion ratio (FCR) = weight of consumed food by fish during the study period (g) / (final body weight (g) – initial body weight (g))

Condition factor (CF): $g/cm^3 = 100$ [final body weight (g) / final body length $(cm)^3$]

Proximate chemical analysis

Proximate composition of the experimental diets and fish body were analyzed according to AOAC method (AOAC, 2000). Briefly, moisture was measured following oven-drying at 105°C to reach a constant weight, and ash through incineration in a muffle furnace at 550°C for 24h. The content of crud protein (N×6.25) and lipid was determined using Kjeldahl analyzer unit (Schott, Werthein, Germany), and ether extraction methods, respectively.

Blood collection and biochemical analysis

At the eighth week (at the end of experimental period), nine fish per treatment were randomly selected and anesthetized using clove powder (170 mg L^{-1}). Blood samples were collected from the caudal vein into heparinized plastic syringes, centrifuged at 3000 rpm for 10 min and plasma samples were kept in a freezer at -20°C until subsequent analyses. The concentration of plasma glucose, total protein were measured using commercial kits (Pars Azmoon, Tehran, Iran). The glucose assay was performed by enzymatic and colorimetric methods (GOD-PAD). Briefly, a total volume of 10 µL of plasma sample was mixed with 1.000 µl of reagent and incubated for 20 min at room temperature, and then the absorbance was measured at 546 nm against the blank (10µl of distilled water mixed with 1.000 µl of reagent). The concentration was calculated using the following equation:

Glucose (mg/dl) = sample B/calibrator B × calibrator concentration

Immunoglobulin M (IgM) level was determined according to the method published by Narayanan (1982). Briefly, the same volume (0.1 ml) of serum sample and 12.0% polyethylene glycol solution (Biosystems Made in Spain) were mixed and incubated for 120 min, and then IgM molecule was precipitated

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following centrifuging at 5000 rpm at 4 °C. The supernatant was diluted 30 times with 0.85% NaCl and the protein content was determined according to Bradford method. The difference between protein values of untreated and polyethylene glycol treated samples was used to determine IgM in the samples. In addition, triglyceride and cholesterol were measured using specific kits (Pars Azmun Co., Iran) based on protocol of manufacturer's instruction (Rastiannasab *et al.*, 2014). Red blood cell count (RBC) and neutrophil count were performed optically with a Neubauer chamber (Merck, Germany).

Intestinal bacterial population

At the end of the nutrition trial, nine fish per treatment (n=3 per replicate) were sampled and their skin was sterilized before opening the abdomen and dissecting their intestine. The intestine samples were pooled, weighed (≈ 1 gr), and homogenized in sterile saline (i.e., physiological serum). The suspensions were serially diluted to 10^{-7} and 100 µL and the solution was spread onto plate count agar (PCA) media to monitor total bacterial count and also onto De Man, Rogosa and Sharpe (MRS) to determine *Lactobacillus* bacteria count. Cultivated plates were incubated at 37°C for 48h and number of colonies was counted.

Statistical analyses

Data analyses were performed using SAS software version 2004. All results are presented as mean ± standard deviation (S.D.). Significant differences were determined using one-way ANOVA, followed by Duncan's test to compare the differences among experimental groups and the respective control group. Differences were considered statistically significant when $p<0.05$.

Results

Molecular weight distribution of CPHs

The hydrolyzed process yielded peptides with different molecular weights (MW) in the range of 180-3000 Da (Table 2). Peptides with molecular weight of 180-500 Da constituted the highest proportion of CPHs (54.89%), predominated by di- and tripeptides, followed by oligopeptides and polypeptides (38.32%; with MW of 500 to>2500 Da), and free amino acids (5.8%, MW<180 Da).

Table 2: The molecular weight distribution of canola protein hydrolysate (%).

Molecular weight (Da)	Obtained peptides (%)
>3000	0.09
2000-3000	0.89
1000-2000	11.63
500-1000	26.69
180-500	54.89
180<	5.81

Growth performance and body composition

Whole-body responses to the experimental diets exhibited significant differences between the experimental groups (Table 3). With increasing concentration of CPH, all of these parameters displayed a similar pattern. FCR and CF showed a CPH dose-dependent decrease and the fish treated

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with CPH_{500} diet demonstrated lowest FCR and CF levels (p<0.05). Interestingly, other indices, including SGR, PER, BWI, standard length (SL), total length (TL), survival rate, and food consumption demonstrated a CPH dose-dependent increase, with highest values in the fish treated with CPH_{500} (p<0.05).

Table 4 shows the whole body proximate composition of the experimental animals. No difference was observed in the percentage of total protein, total lipid, ash, and moisture contents between the control group and the CPH-treated groups (p≥0.05).

Table 3: Growth performance indices of beluga (*Huso huso*) juveniles fed with diets containing different levels (0, 300, 400, and 500 mg per kg diet) of canola protein hydrolysate (CPH). Data are mean ± SD. Columns sharing the same alphabet are not significantly different (p>0.05).

Experimental diets	Indices								
	Survival (%)	Food consumption (g)	FCR	CF	SGR (%)	PER	WG (%BWI day^{-1})	Standard length (cm)	Total length (cm)
CPH_0 (basal diet)	63	17656.60	1.06±0.03^{a}	0.48±0.01^{a}	3.95±0.01^{a}	1.76±0.05^{c}	248.44±2.9^{c}	33.89	37.80
CPH_{300}	64	17890.00	0.96±0.05^{b}	0.45±0.03^{ab}	4.04±0.08^{b}	1.91±0.11^{b}	264.79±4.74^{b}	33.90	40.10
CPH_{400}	68	18022.02	0.95±0.02^{b}	0.42±0.00^{bc}	4.09±0.02^{b}	2.03±0.04^{b}	270.53±3.68^{b}	33.32	41.03
CPH_{500}	69	18455.69	0.87±0.01^{c}	0.40±0.01^{c}	4.23±0.01^{a}	2.16±0.03^{a}	293.07±2.16^{a}	35.84	43.08

Table 4: Body composition (moisture, lipid, protein and ash) of beluga (*Huso huso*) juveniles fed on diets supplemented with different levels of canola protein hydrolysate (0, 300, 400, and 500 mg kg^{-1}; denoted as CPH_0, CPH_{300}, CPH_{400}, CPH_{500}, respectively) for 8 weeks. Data are mean±SD.

Approximate analysis	Experimental diets			
	CPH_0 (basal diet)	CPH_{300}	CPH_{400}	CPH_{500}
Protein (% DM)	19.02±0.12	18.85±0.21	18.97±0.16	18.95±0.21
Fat (% DM)	11.48±0.22	11.85±0.30	11.36±0.39	11.89±0.41
Ash (% DM)	2.80±0.06	2.45±0.03	2.13±0.01	2.36±0.02
Moisture (%)	65.22±6.32	65.73±7.41	65.45±7.21	65.33±5.63

Biochemical and immunological indices

Effects of the experimental diets on some blood biochemical and immunological indices are shown in Table 5. Triglyceride, cholesterol, and glucose significantly decreased in all of the fish that received CPH when compared to the control group (p<0.05). Number of RBC showed a CPH-concentration dependent increase, with the highest level in the fish fed with CPH_{500} diet, whereas number of white blood cells (WBC) reduced, especially in the fish treated with CPH_{400} and CPH_{500} (p<0.05). IgM demonstrated a U-shape response following increasing concentration of CPH in the diets (p<0.05).

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Table 5: Biochemical, hematological, and immunological parameters of beluga (*Huso huso*) juveniles fed with diets supplemented with different levels (0, 300, 400, and 500 mg per kg diet) of canola protein hydrolysate (CPH). Data are mean ± SD, n=3. Columns sharing the same alphabet are not significantly different (p>0.05).

Treatments	Indices					
	Triglyceride (mg/dl)	Cholesterol (mg/dl)	Glucose (mg/dl)	RBC×10^6 (mm3)	WBC×10^3 (mm3)	IgM (mg/dl)
CPH_0 (basal diet)	203.4±0.66^a	92.6±13.00^c	106.3±17.3^a	0.43±0.06^a	19.88±1.0^b	50.1±3.40^a
CPH_{300}	155.3±0.60^a	54.9±10.00^b	89.4±7.9^a	0.49±0.03^a	19.48±1.4^b	39.2±5.27^{ab}
CPH_{400}	172.3±0.49^b	69.8±9.50^a	91.5±8.4^b	0.53±0.10^a	28.50±1.5^a	40.3±4.13^b
CPH_{500}	99.4±0.44^b	53.0±6.04^a	76.8±5.4^b	0.55±0.10^a	29.96±2.6a	95.2±5.87^b

Microbial analysis

At the end of the feeding trial, intestinal population of *Lactobacillus* spp. and total bacteria enhanced significantly in all experimental fish groups fed with CPH-supplemented diets (p<0.05; Fig. 1). The fish fed with CPH_{500} showed the highest total bacterial population (2.1×10^6 CFU) and *Lactobacillus* spp. (1.6×10^4 CFU).

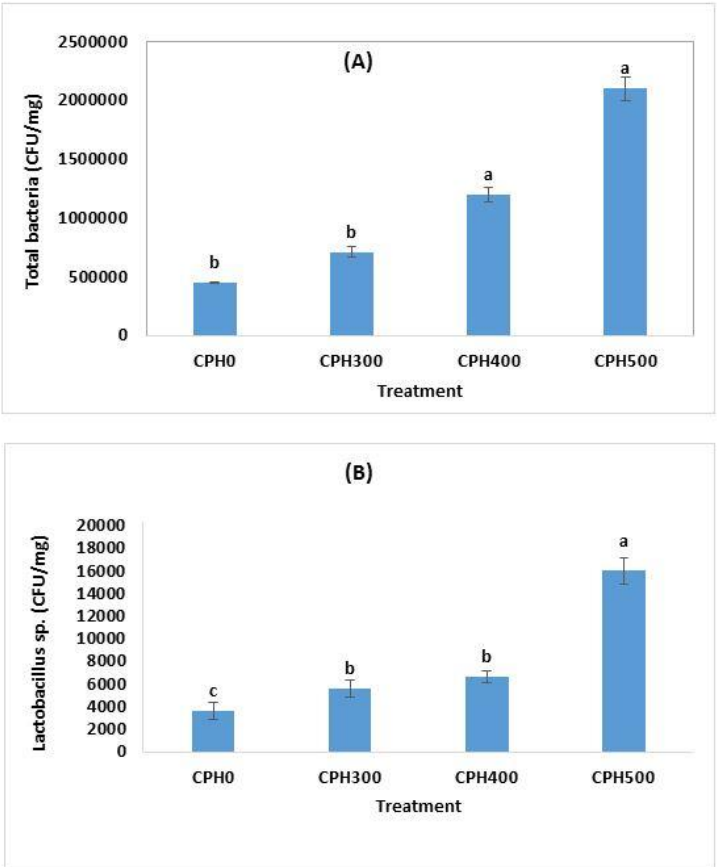

Figure 1: Intestinal loads of total bacteria (A) and *Lactobacillus* spp. (B) in beluga (*Huso huso*) juveniles fed with diets supplemented with different levels of canola protein hydrolysate (0, 300, 400, and 500 mg kg^{-1} diet; denoted as control, CPH_{300}, CPH_{400}, and CPH_{500}). Data are mean and error bars are SD. Bars sharing the same alphabet are not significantly different (p>0.05).

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Discussion

The current study investigated the effects of dietary canola hydrolyzed peptides on growth performance, blood biochemical and immunological indices, and intestinal bacterial population of beluga sturgeon (*Huso huso*) juveniles. Molecular weight distribution of CPHs confirmed that canola proteins were sufficiently hydrolyzed and peptide fractions with different sizes and molecular weights were produced, with about 55% being of di- and tripeptide fractions. In this study, non-specific serum safety parameters including total protein, immunoglobulin (IgM), and albumin and complement system were measured. Canola hydrolyzed protein increased total protein; immunoglobulin (IgM), albumin, and serum complement system. In other studies, consumption of canola meal protein increased serum albumin in indigo tilapia (Zhou and Yue, 2010). This finding suggests higher bioavailability of peptides in digestive system of the experimental fish.

In the present research, all of the fish that received dietary CPH (especially CPH_{500}) showed a CPH dose-dependent response concerning growth performance indices (SGR, FCR, CF, BWI, SL, and TL) as well as survival rate and food consumption. Hydrolyzed peptides can provide juveniles nutritional requirements. improving digestive system. These observations could be attributed to higher absorption of hydrolyzed peptides and, in turn, better palatability of the supplemented diets (Ostaszewska *et al.*, 2010). These findings corroborate the observations of Ostaszewska *et al.* (2010) who reported significantly higher growth performance of rainbow trout (*Oncorhynchus mykiss*) following feeding with dipeptide- and free amino acid-supplemented diets than those received intact protein. A supportive opinion is that high amounts of glutamic amino acid in plant hydrolyzed proteins could improve the taste and aroma of foods and thereby enhance their absorption within the digestive system (Pasupuleti, 2006).

Blood biochemical and immunological indices are of high importance for indicating physiological and general health status of fish, reflecting nutritional and environmental changes (Gui *et al.*, 2010). Regarding the role of peptide in improving immune system, the canola protein hydrolysate can lead to lowering blood glucose and cortisol levels. Concentrations of triglyceride, cholesterol, and glucose decreased in the fish treated with CPH, and these results accord with earlier research which showed that using 100% hydrolyzed soybean protein (Song *et al.*, 2014), fish protein hydrolysate (Hevrøy *et al.*, 2005; Xu *et al.*, 2016), and cottonseed meal protein hydrolysate reduced the concentration of cholesterol and triglyceride significantly (Gui *et al.*, 2010). Number of RBC showed a CPH concentration-dependent increase. This finding differs from a previously published study in which Atlantic salmon (*Salmo salar* L.) fed with fish protein hydrolysate-supplemented diet showed no change in RBC number (Hevrøy *et al.*, 2005). However, a possible explanation for this might be

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that CPH stimulated erythropoiesis in treated fish. Previous studies demonstrated that immunostimulants can enhance non-specific immune indices such as plasma IgM (Ronyai *et al.*, 1990; Bagni *et al.*, 2000; Pourgholam *et al.*, 2016). In the current study, innate immune parameters (WBC and IgM) revealed different responses. Number of WBC was reduced, especially in CPH_{400} and CPH_{500} treatments whereas IgM level showed a U-shape response. These data are dissimilar to earlier findings by Song *et al.* (2014) who found significant increase of WBC in juvenile starry flounder (*Platichthys stellatus*) after dietary replacement of fishmeal with soy protein hydrolysates. This can be explained due to difference among fish species, different methodologies including different nutritional requirements, sources of dietary protein, other dietary components, digestibility of various dietary components, difference among growth stages of fish species, and the difference in ability of fish to mount successful immune responses, experimental conditions, and statistical methods used among the studies. These rather contradictory results may be due to different peptide fractions of CPH and soy protein hydrolysates as well.

Inclusion of hydrolyzed peptides in fish diet could alter their digestive microbiota. Therefore, it is particularly important to monitor intestinal microbiota to make sure that hydrolyzed peptides are not detrimental to positive bacterial community, especially probiotics of digestive tract (Egerton *et al.*, 2020). Previous research demonstrated that lactic acid bacteria increased in fish intestine following being fed with plant-based diets (Hartviksen *et al.*, 2014; Gajardo *et al.*, 2017). Observed increase of *Lactobacillus* spp. and total bacteria in intestine of the treated fish suggested that CPH could act as prebiotics and induce proliferation of intestinal endemic probiotics of the experimental fish. These data are parallel with those observed in earlier studies (Agyei and Danquah, 2011; Karimzadeh *et al.*, 2016, 2017).

This research is undertaken to evaluate feasibility of CPH inclusion in diet of beluga juveniles through analyzing some growth performance indices, blood biochemical and immunological indices, and intestinal microbiota. Generally, it is found that CPH-supplemented diets could improve the growth, immunity, and gastrointestinal microbial population of beluga juveniles, particularly CPH_{500}. Taken together, CPH could be introduced as an alternative for fishmeal in beluga diet, although studies need to be performed to elucidate the underlying mechanisms by which CPH improves the fish growth.

Acknowledgment

The authors gratefully acknowledge the support of Islamic Azad University, Babol Branch. Our appreciation is extended to “Hamid Salari Joo” for his Academic English Language Editing

[DOR: 20.1001.1.15622916.2021.20.4.18.3]

Service during preparation of the manuscript. Last but not least, the author heartfully says thanks to Mr. Islami the manager of Gharehbrun Company, a sturgeon breeding farm in Iran, for providing the experimental condition and fish.

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