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RESEARCH ARTICLE

A survey of the functional, gut digestive, and serum antioxidant factors in *Salmo trutta caspius* (Kessler) fingerlings with the application of a dietary synbiotic

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Abstract. This study evaluated the effectiveness of BetaPlus® combined with isomalto-oligosaccharide (IMO) in Caspian brown trout, *Salmo trutta caspius* (Kessler), fingerlings. A total of 120 Caspian brown trout (8.75 ± 0.03 g) were fed in two treatments, including the control diet and the synbiotic diet (0.1% BetaPlus® + 0.2% IMO) in three replicates per treatment for seven weeks. The growth indices (final weight, weight gain, average daily growth, specific growth rate, feed efficiency, and protein efficiency ratio) exhibited significant improvement in the fish fed the synbiotic diet ($P < 0.05$). The highest ash crude protein, and crude fiber, as well as the lowest crude lipid, dry matter, and carbohydrate detected in the carcass of fish treated with the synbiotic were significant ($P < 0.05$). In addition, the fish fed the synbiotic diet showed significantly higher gut trypsin activity and trypsin:chymotrypsin ratio, as well as serum superoxide dismutase activity ($P < 0.05$). Thus, BetaPlus® in combination with IMO can effectively lead to a considerable increase in functional factors, as well as gut proteases and serum antioxidant indicators in *S. trutta caspius* fingerlings.

Keywords: antioxidant defense, carcass, Caspian brown trout, growth performance, proteases, synbiotic

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Introduction

The Caspian brown trout (*Salmo trutta caspius* Kessler) (family: Salmonidea) is one of the nine brown trout (*Salmo trutta*) subspecies with the highest weight, size, and growth rate, and it is one of the most important endemic species of the Caspian Sea southern coast (Dorafshan et al. 2008). During two past decades, natural stocks of this valuable anadromous species have increasingly decreased due to human activities, including overfishing, habitat pollution, and environment alterations (Sotoudeh et al. 2011). According to the International Union for Conservation of Nature (IUCN) criteria, the Caspian

brown trout is critically endangered in the Caspian sea southern coast (Coad 2000). Therefore, this very vulnerable species is under protection of the Iranian Fisheries Organization (IFO).

Over the past two decades, aquaculturists have used antibiotics widely to improve growth performance, feed efficiency, immune stimulation, and the survival rate of aquatic animals against environmental stress and diseases. Because of the accumulation of antibiotic residues and increasing pathogen resistance in different animals (Ramos et al. 2013), there is currently worldwide consideration of using alternative feed supplements such as biotic components like probiotics, prebiotics, and synbiotics in aquatic production. As defined by Ringø et al. (2010), probiotics are live microorganisms added to feed or rearing water that when administered to fish in adequate amounts confer increase in viability, enhance immune and digestive systems, promote growth and general welfare. According to the definition of Gibson and Roberfroid (1995), prebiotics are as non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, and thus improves host health. It is well documented that the addition of prebiotics to probiotics compounds as synbiotics thanks to the synergistic effects that can result in probiotic bacteria survival and growth enhancement (Adebola et al. 2014).

BetaPlus®, the commercial probiotic used in this research, is a two-species *Bacillus* probiotic that includes *Bacillus subtilis* and *B. licheniformis*. Isomalto-oligosaccharide (IMO), which was the prebiotic applied in our study, is also called a bifidus-factor because of its augmentation of gut acid lactic bacteria, or bifidobacteria (Ringø et al. 2010). Furthermore, Kaneko et al. (1995) state that IMO is mainly composed of isomaltose, isomaltotriose, panose, isomaltotetraose, etc. Several studies demonstrated that different synbiotic compounds can enhance growth, immunity, and antioxidant defense in fish species (Zhang et al. 2013, 2015, Van Doan et al. 2016, Hoseinifar et al. 2017). This study was preliminary designed to evaluate the efficiency of a dietary synbiotic (BetaPlus® + IMO) on growth enhancement, carcass alteration, antioxidant defense, and gut digestive enzymes in *S. trutta caspius* fingerlings.

Material and method

Fish and trial condition

At the start of the trial, 120 Caspian brown trout, *Salmo trutta caspius* fingerlings (8.75 ± 0.03 g) were stocked into six polypropylene tanks (300 l) (20 fish per tank) at the health and disease section of the Coldwater Fish Research Center (CFRC) (Dohezar, Tonekabon, Mazandaran, Iran). For acclimation to experimental conditions, the fish were fed a commercial extruded diet for two weeks. The water in each tank was continuously aerated through air stones. The trial was performed in a flow-through system in a 12:12 h light:dark cycle. Based on the previously published study by Aftabgard et al. (2017), the Caspian brown trout fingerlings were fed the trial diets manually to apparent satiation two times a day for seven weeks in two trial groups, including a control group and a synbiotic group (0.1% BetaPlus® + 0.2% IMO) with three replicates per group.

Feed preparation

In this study, a commercial extruded diet (basal or control diet) containing 46% crude protein, 14% crude lipid, 3% crude fiber, 10% ash, 11% moisture, and 1.5% phosphorous was purchased from Faradaneh Co. (Iran). BetaPlus® is a commercial probiotic containing two bacteria strains in spore form (5.12×10^{12} CFU kg $^{-1}$ of each strain) of *B. subtilis* (DSM 5750) (obtained by soya bean fermentation) and *B. licheniformis* (DSM 5749) (isolated from soil) that was purchased from the Biochem Co. (Germany). The prebiotic IMO (Serva Feinbiochemica Co., Heidelberg/New York) was obtained from chicory. To prepare the synbiotic diet, BetaPlus® at 0.1% and IMO at 0.2% of the basal diet

were blended, dissolved in sterile distilled water (100 mL per 1 kg of diet) (Adel et al. 2017), and then sprayed on the extruded feed. The control diet was sprayed with sterile distilled water only. Then, according to the Aftabgard et al. (2017) study, the trial diets were air-dried and stored until use. The trial diets were then air-dried for 4 h and stored at 4°C until use. In order to maintain the quality of the food, the trial diets were prepared twice weekly.

Growth and carcass indices assay

At the end of seven weeks and after 24 h fasting, the Caspian brown trout in two trial treatments were randomly sampled, and anesthetized with clove powder at 200 mg L^{-1}. Thus, four fish per replicate (12 fish per treatment) were weighed individually. The following formulas were used to calculate the growth parameters:

- weight gain, WG, (g $fish^{-1}$) = final body weight (g) – initial body weight (g);
- daily growth rate, DGR (g d^{-1}) = (final body weight (g) – initial body weight (g)) × rearing $period^{-1}$ (days);
- specific growth rate, SGR (% d^{-1}) = 100 × (ln final body weight (g) – ln initial body weight (g)) × rearing $period^{-1}$ (days);
- feed efficiency, FE (%) = (wet weight gain (g) × dry feed $intake^{-1}$ (g)) × 100
- protein efficiency ratio, PER = (final fish weight (g) – initial fish weight (g)) × quantity of protein fed^{-1} (g).

After measuring the final weight, carcass analysis of the four fish per triplicate was performed in accordance with the AOAC (1990). Crude protein content was assessed with the Kjeldahl method. Crude lipid was analyzed using the Soxhlet method. Crude fiber was measured using an automatic analyzer. Ash content was determined with cremate in an electric oven at 550°C for 4 h. Dry matter was estimated gravimetrically after drying the samples at 105°C for 24 h. Carbohydrate content was determined according to the Gouveia and Davies (2000) method.

Blood sampling and serum antioxidant enzyme assay

To evaluate the serum antioxidant enzymes, blood samples were collected from five Caspian brown trout per replicate anesthetized with 200 mg L^{-1} clove powder by cutting of the caudal vein and pooling the blood into Eppendorf tubes. Serum samples were obtained using standard methods, and kept at -20°C until analysis. To measure the serum superoxide dismutase (SOD) activity, the pyrogallol auto-oxidation reaction in the presence of hydrogen peroxide (H_2O_2) was investigated. Thus, SOD activity decreased to inhibit pyrogallol auto-oxidation and was measured based on the decrease in light absorption with a spectrophotometer at 420 nm (Marklund and Marklund 1974). To assess the catalase activity (CAT), the serum samples were placed in the presence of an H_2O_2 solution for 10 minutes (at room temperature). An ammonium molybdate solution was used to stop the oxidation process. Because of the reaction of ammonium molybdate and H_2O_2, a yellow compound was formed. Thus, CAT activity was determined based on the absorption of the yellow colour at 410 nm with a spectrophotometer against the control (blank) (distilled water was used as the blank) (Goth 1991).

Gut digestive enzymes activity assay

To determine the activities of digestive enzymes, intestinal tissue samples were collected from three Caspian brown trout under 24 h food interruption per tank. At first, according to Guzmán-Villanueva et al. (2014), intestinal samples were weighed separately and homogenized in four volumes of cold distilled water (4°C). Then, the suspensions were centrifuged at 12000 g for 10 min at 4°C, and stored at -80°C until enzymatic analysis. The amylase activity was determined based on the method described by Bernfeld (1955), using starch as the substrate. The lipase activity was measured according to the Yanbo and Zirong (2006) protocol by evaluating the fatty acids produced by the hydrolysis process of triglycerides in a stabilized emulsion of olive oil. The trypsin activity

was assayed according to the method described by Erlanger et al. (1961) using N-a-benzoyl-DL-arginine 4-nitroanilide hydrochloride (BAPNA) as a substrate in 10 mM dimethyl sulfoxide (DMSO) and a 50-mM Tris–HCl buffer with 10 mM $CaCl_2$ at pH 8.2. Chymotrypsin activity was assayed according to the procedure explained by Del Mar et al. (1979) using N-succinyl-Ala-Ala-Pro-Phe-p-nitroanilide (SAAPNA) as the substrate in 10 mM DMSO and 100 mM Tris–HCl buffer with 10 mM $CaCl_2$ at pH 7.8. According to Bradford (1976), the supernatant soluble protein content was estimated using bovine albumin as the standard solution. The digestive enzyme activity in the current trail was expressed as U mg^{-1} protein min^{-1}. The above enzymatic analysis was conducted at 25°C in triplicate.

Statistical analysis

The variance homogeneity of all the data was first checked with the Kolmogorov-Smirnov test and then exposed to independent sample *t*-tests to compare two treatments via SPSS 23.0 (SPSS Inc., Chicago, IL, USA). The average values were significant by $P < 0.05$. The data for all the assays are also presented as average values ± standard error of the mean (SEM).

Results

After seven weeks of the feeding trial, the growth parameters (final weight (FW), WG, DGR, SGR, FE and PER) of fish fed the BetaPlus® + IMO diet were significantly higher than those of the fish fed the control diet ($P < 0.05$; Table 1). The carcass crude protein, ash, and crude fiber were significantly higher in the fish fed the BetaPlus® + IMO diet than the control group ($P < 0.05$), while the other carcass items such as crude lipid, dry matter, and carbohydrate were significantly lower in the fish fed the synbiotic diet than in the fish fed the control diet ($P < 0.05$; Table 2). The effects of the

Table 1
Growth factors of Caspian brown trout fingerlings fed the trial diets for seven weeks

	Treatments	
Parameters	Control	BetaPlus® + IMO
Final weight (g)	16.32 ± 0.73^{b}	18.67 ± 0.24^{a}
Weight gain (g $fish^{-1}$)	8.19 ± 0.97^{b}	10.57 ± 0.45^{a}
Specific growth rate (% day^{-1})	1.28 ± 0.11^{b}	1.58 ± 0.05^{a}
Daily growth rate (g day^{-1})	0.17 ± 0.02^{b}	0.22 ± 0.01^{a}
Protein efficiency ratio	1.51 ± 0.31^{b}	2.25 ± 0.07^{a}
Feed efficiency (%)	66.33 ± 13.57^{b}	98.67 ± 3.18^{a}

Values (mean ± SEM) in each row with different superscript notations indicate statistically significant differences between the treatments ($P < 0.05$)

Table 2
Carcass composition of Caspian brown trout fingerlings fed the trial diets for seven weeks

	Treatments	
Parameters	Control	BetaPlus® + IMO
Crude protein (%)	54.96 ± 0.48^{b}	62.91 ± 0.15^{a}
Crude lipid (%)	26.05 ± 0.21^{a}	21.02 ± 0.65^{b}
Crude fiber (%)	0.33 ± 0.03^{b}	0.60 ± 0.02^{a}
Ash (%)	6.89 ± 0.02^{b}	8.04 ± 0.10^{a}
Dry matter (%)	29.13 ± 0.35^{a}	26.96 ± 0.44^{b}
Carbohydrate (%)	10.64 ± 0.46^{a}	6.86 ± 0.21^{b}

Values (mean ± SEM) in each row with different superscript notation indicate statistically significant differences between the treatments ($P < 0.05$)

Table 3
Serum antioxidant enzyme activity of Caspian brown trout fingerlings fed the trial diets for seven weeks

	Treatments	
Parameters	Control	BetaPlus® + IMO
SOD (μ ml^{-1})	52.00 ± 4.73^{b}	72.67 ± 5.90^{a}
CAT (μ ml^{-1})	4.37 ± 0.12^{a}	4.07 ± 0.09^{a}

Values (mean ± SEM) in each row with different superscript notation indicate statistically significant differences between the treatments ($P < 0.05$)

Table 4
Digestive enzyme activity and soluble protein concentration in the gut of Caspian brown trout fingerlings fed the trial diets for seven weeks

	Treatments	
Parameters	Control	BetaPlus® + IMO
Soluble protein (mg ml^{-1})	1.86 ± 0.29^{a}	2.42 ± 0.11^{a}
Amylase (U mg^{-1} protein)	43.51 ± 12.24^{a}	49.10 ± 9.25^{a}
Lipase (U mg^{-1} protein)	875.53 ± 106.25^{a}	857.02 ± 125.35^{a}
Trypsin (U mg^{-1} protein)	11.19 ± 0.33^{b}	29.92 ± 0.63^{a}
Chymotrypsin (U mg^{-1} protein)	25.58 ± 0.73^{a}	29.49 ± 2.79^{a}
TR:CH ratio	0.44 ± 0.01^{b}	1.03 ± 0.09^{a}

Values (mean ± SEM) in each row with different superscript notation indicate statistically significant differences between the treatments ($P < 0.05$)

synbiotic (BetaPlus® + IMO) on the serum antioxidant responses of Caspian brown trout are shown in Table 3. The serum SOD activity was significantly higher in the fed fish the synbiotic than in the control fed fish ($P < 0.05$). However, the serum CAT was non-significantly higher in the synbiotic group than in the control group ($P > 0.05$). The analysis of the intestinal enzymes activity of Caspian brown trout fed the experimental diets is displayed in Table 4. After the seven-week trial period, the gut trypsin activity and trypsin-chymotrypsin ratio (TR:CH) were significantly higher in the fish treated with BetaPlus® + IMO ($P < 0.05$). The gut amylase and chymotrypsin activities and the soluble protein concentration were non-significantly higher in the Caspian brown trout fed the synbiotic diet ($P > 0.05$), while a non-significant decrease of gut lipase activity was observed in the synbiotic treatment ($P > 0.05$).

Discussion

Recently, using synbiotics as an alternative to antibiotics by aquatic nutritionists has been expanded to improve the speed and levels of growth, reduce the costs of production, and increase safety against stress and a variety of pathogens in aquaculture farms. Based on our findings (Table 1), the significantly enhanced growth function in Caspian brown trout fingerlings fed the synbiotic (BetaPlus® + IMO) diet could be due to improved efficiency in feed consumption and feed intake compared to the fish fed the control diet. Previous findings in studies that used other synbiotics were similar to the results of the present research and improved growth performances have been reported by Rodriguez-Estrada et al. (2013) in rainbow trout, *Oncorhynchus mykiss* (Walbaum) fed a combined diet of *Enterococcus faecium* and mannan oligosaccharide (MOS), and by Li et al. (2009) in white shrimp (*Litopenaeus*

vannamei) fed a diet containing *Bacillus* OJ and IMO.

The antioxidant defense system of fish might be associated with factors such as feeding behavior, feed consumption, age, and diet type (Martinez-Alvarez et al. 2005). The combined inclusion of BetaPlus® and IMO significantly increased the activity of serum SOD in Caspian brown trout compared with the control group, which can be caused by the effective role of the dietary synbiotic in regulating the balance of the body's free radicals and enhancing antioxidant capacity, which resulted in improved host immunity (Lee et al. 2013). In accordance with our results, Black Amur bream, *Megalobrama terminalis* (Richardson) fed a combined diet of *B. licheniformis* and fructooligosaccharide (FOS) revealed significantly increased serum SOD activity (Zhang et al. 2013).

The two key digestive proteases of trypsin and chymotrypsin are effective indicators in the nutritional performance of the fish species. Additionally, the TR:CH ratio can be considered to be an influential indicator of nutritional performance in fish (Cara et al. 2007). In our study, significant improvements in gut trypsin activity, the TR:CH ratio, and a marginal increase in gut chymotrypsin activity using the combined inclusion of BetaPlus® and IMO in the diet can enhance feed digestion and absorption, and, as a result, the growth efficiency of Caspian brown trout fingerlings, as was observed in the present research. Similarly, the inclusion of the synbiotic Biomin® IMBO at a level of 1 g kg^{-1} to the diet of common carp, *Cyprinus carpio* L., fingerlings showed the highest significant activity of trypsin and chymotrypsin enzymes compared to the control fish (Ghasempour Dehaghani et al. 2015).

It is evident that the value of product in aquaculture is associated with the amounts of body protein and lipid; thus, many previous nutritional studies have focused on these two carcass factors by enhancing the nutrient intake and consequently inducing better growth performance and carcass quality. Based on our data, the crude protein and lipid contents of the carcass were significantly affected by the dietary synbiotic (BetaPlus® + IMO). In contrast to our results, carcass composition was not modified in rainbow trout fed a dietary mixture of *E. faecalis* and MOS (Rodriguez-Estrada et al. 2013), nor in Black Amur bream following the combined incorporation of *B. licheniformis* and FOS in the feed (Zhang et al. 2015). Similar to the achievements of this study, the carcass crude protein and lipid were significantly increased and decreased, respectively, in Japanese flounder, *Paralichthys olivaceus* (Temminck & Schlegel) following combined supplementation with *B. clausii* and FOS, MOS (Ye et al. 2011). Grisdale-Helland et al. (2013) states that the body reduced lipid is often accompanied by an optimal balance of amino acids, which can usually be associated with reduced catabolism of amino acids, and, thus, improved muscle protein synthesis. This phenomenon explains the significant increase and decrease of carcass protein and lipid, respectively, effected by the dietary combination of BetaPlus® and IMO in the diet of Caspian brown trout fingerlings, which is mentioned in the results. The role of minerals in the skeletal structure and in regulating osmotic pressure in the fish body, and especially proteinization processes, is of particular importance during the early stages of fish growth (Lovell 1989). In the present study, the highest amount of carcass ash in the synbiotic treatment indicated an increase in carcass minerals compared to the control group, which was probably due to the effect of the IMO combined with BetaPlus® in increasing the concentration of short-chain fatty acids and, as a result, increasing the absorption of bivalent cations such as calcium, magnesium, zinc, and iron from the gut of the Caspian brown trout fingerlings. This potential effect has been proven for prebiotics (Delzenne and Roberfroid 1994). As non-digestible carbohydrates, or fiber, the presence of IMO in the synbiotic composition applied in the present study also partly explains the carcass composition tendency of the fish given the synbiotic treatment to having significantly increased crude fiber versus significantly reduced carcass carbohydrate in comparison to the control group. Changes in protein density and amino acids are good indicators of changes in the amount of dry matter; thus, increasing carcass protein can lead to reductions in the amount of dry matter in the body (Aletor

et al. 2000); this is also apparent in the carcasses of the Caspian brown trout fingerlings fed the synbiotic diet in the current study.

To conclude, BetaPlus® containing two strains of *Bacillus* bacteria, including *B. subtilis* and *B. licheniformis* at 0.1%, in combination with IMO at 0.2% effectively improved the growth indices, serum antioxidant defense with an emphasis on SOD activity, digestive proteases efficiency (gut trypsin activity and TR:CH ratio), as well as the muscle protein and ash of Caspian brown trout, *S. trutta caspius*, fingerlings. Thus, the synbiotic (BetaPlus® + IMO) is a suitable candidate for enhancing the quality and commercial value of the final product of Caspian brown trout and even other salmonids in aquaculture.

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