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ORIGINAL ARTICLE

Effects of two dietary exogenous multi-enzyme supplementation, Natuzyme® and beta-mannanase (Hemicell®), on growth and blood parameters of Caspian salmon (*Salmo trutta caspius*)

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Abstract Recent increases in feed ingredient costs have motivated the fisheries industry to identify technologies that will improve feed utilisation and reduce the cost per pound of gain. The effects of two supplemental exogenous enzymes (Natuzyme® and Hemicell®) on the growth performance in Caspian salmon (*Salmo trutta caspius*) were examined over an 8-week feeding trial. After the experimental period, the survival rate ranged from 91.33±1.15 % in controls to 96.67±1.15 % in the group that received 0.5 g Natuzyme® kg^{-1}+0.5 g Hemicell® kg^{-1} (NH) in their diet and there was a statistical difference between experimental and control groups ($p<0.05$). Growth rate was significantly higher in the NH group (1.01±0.01) than the other groups (Sig.=0.00). The best feed conversion rate (0.64±0.01) was in the NH group and it was significantly lower than the control group, the 0.5 g Natuzyme® kg^{-1} group, and the 0.25 g Hemicell® kg^{-1} group (Sig.=0.03). The best final body weight (80.68±5.27) was observed in the NH group. Also, WBC count (7,716.67±348.80 N/mm^3) was significantly higher in the NH group compared to the control (6,916.67±194.10 N/mm^3; $p<0.05$). No difference was observed in haematocrit%, haemoglobin, red blood cell, mean corpuscular volume, mean corpuscular haemoglobin, and mean corpuscular haemoglobin concentration ($p>0.05$). The results suggested that enzyme supplementation caused significant improvement on growth performance and feed utilisation in Caspian salmon.

Keyword Caspian salmon · Enzyme · Supplementation · Haematology · Growth

Introduction

The identification and alleviation of factors that inhibit nutrient utilisation are necessary for successful feed fish production. Commercial fish feeds usually contain high fish meal content ranging from 30 to 50 %, but because of high cost and its scarcity, this component is generally omitted in the feed regions (Goda et al. 2007; Davies and Gouveia 2008). Hence, aquaculture nutrition has been trying to improve the nutritional value of fish feed by enzyme supplementation in the last decades. Exogenous enzymes are now extensively used throughout the world as additives in animal diets. However, the effects of exogenous enzymes can be variable and are dependent on a large number of factors such as the age of animal and the quality and type of diet (Bedford and Schulze 1998; Acamovic 2001). Supplementation with enzymes is effective to eliminate the anti-nutritional factors and improve the utilisation of dietary energy and amino acids, resulting in improved fish performance (Farhangi and Carter 2007; Lin et al. 2007; Soltan 2009). Supplementing fish feed with an enzyme or enzyme mixture that possess a broad-spectrum range of activities may improve the digestibility

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and as a result, growth performance in several cultured fish species like channel catfish (Jackson et al. 1996), *Pangasius pangasius* (Debnath et al. 2005), *Clarias batrachus* and *Clarias gariepinus* (Giri et al. 2003), tilapia (Drew et al. 2005) and salmon (Refstie et al. 1999; Odetallah et al. 2005). The digestibility of all nutrients, however including carbohydrates, protein, and minerals seems to be affected by exoenzymes (Felix and Selvaraj. 2004).

Hemicell® is a fermentation product of *Bacillus lentus*; its active ingredient is β-mannanase, which hydrolyzes β-mannan (Zou et al. 2006). The use of exogenous enzymes, such as xylanase, α-galactosidase, β-glucanase and endo-β-mannanase, to make oligosaccharide fraction more digestible has shown positive results in some terrestrial species such as poultry (Lobo 1999).

Haematological techniques, including erythrocyte count, haemoglobin concentration, haematocrit and leucocyte count, have provided valuable knowledge for fishery biologists in the evaluation of fish health and in monitoring stress responses and white blood cell counts can be applied as a measure of general immune response (Blaxhall 1972; Soivio and Oikari 1976).

Caspian salmon (*Salmo trutta caspius*), a valuable species in the Caspian Sea, which has unfortunately become endangered due to overharvesting, dam construction on rivers, and urban and agricultural pollution, is cultured in order to protect from extinction.

The objective of this study was to investigate the effect of a commercially prepared exogenous multi-enzyme complex Natozyme® and Hemicell® on growth, feed utilisation, survival and some blood parameters in Caspian salmon *S. trutta caspius*.

Method and material

Experimental fish

Four hundred fifty fish (initial mean weight, 24 g), kept in Shahid Bahonar Fisheries Research. Units were randomly allocated into 550 L fibre glass tanks at a density of 50 fish per tank. The tanks were equipped with aeration and supplied with continuously flowing water (2 L min^{-1}).

Experimental diets

Caspian salmon were fed with trout diets (Behparvar, Iran: 48 % protein, 18 % lipid).

(a) 0.25 g Natuzyme® kg^{-1} and 0.5 g Natuzyme® kg^{-1}, was incorporated into the trout diet to formulate two experimental diets named N1 and N2, respectively
(b) 0.25 g Hemicell® kg^{-1} and 0.5 g Hemicell® kg^{-1}, was incorporated into the trout diet to formulate two experimental diets named H1 and H2, respectively
(c) 0.5 g Natuzyme kg^{-1}+0.5 g Hemicell kg^{-1} was incorporated into the trout diet to formulate experimental diet named NH
(d) Control diet was unmodified trout diet free from any multi-enzyme (0.0 g Natuzyme®+0.0 g Hemicell® kg^{-1})

Multi-enzyme complex Natuzyme® (Bioprotin, Australia), contains protease, lipase, fitase, α amilase, cellulase, amiloglucosidase, β-glucanase, pentosonase, hemicellulase, xylanase, pectinase, acid phosphatase and acid phytase while multi-enzyme complex Hemicell® contains Endo-β-mannanase, amylase, xylanase, cellulose and α-galactosydase.

To prepare the experimental diet, the multi-enzyme complexes were mixed with oil and sprayed onto the trout diet. The control diet was also sprayed with oil. These diets were used after drying in the open air for 2 h.

Experimental procedure

Each of the six experimental diets was randomly assigned to triplicate groups of fish and all the groups were fed with their respective diet to 3 % body weight day twice daily for 8 weeks. Also, water parameters were recorded every week and dissolved oxygen and pH were found to be 7.5–8.5 and 7.8–8.2 mg L^{-1}, respectively, throughout the study. Fish in all groups were counted and group weights were taken for estimation of weight gain and growth parameters.

Calculation formula for relative index

The initial and final weights of fish in each group were measured individually. Specific growth rate (SGR), condition factor (CF), feed conversion rate (FCR), growth rate (GR) and survival rate (SR) were calculated according to Laird and Needham (1988) as follows:

1. FCR = weight gain/feed consumption
2. SGR = 100(Ln(average terminal BW)−Ln(average initial BW)/test days)
3. Percentage body weight gain = ((final weight−initial weight)/initial weight)×100
4. CF = weight (g)/[length (cm)]^3×100
5. SR = number of fish at end of test/number of fish on first day of test×100
6. GR = body weight final−body weight initial/test days

Blood samples and haematological assay

On the first day and at the end of the experimental period (eighth week), 10 fish were randomly selected from each group for haematological assay. Fish were anaesthetized with 25 ppm clove powder, and blood samples were taken from the caudal vein. Haematological parameters were

evaluated as follows. Haematocrit was determined by the micro-haematocrit technique using capillary tubes by centrifugation at 12,000×*g* for 6 min. Haemoglobin (Hb) was determined spectrophotometrically (540 nm) using the cyanomethaemoglobin method. Red blood cell (RBC) counts were also estimated (Hesser 1960). Mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC, percent) and mean corpuscular volume (MCV, cubic micrometer) were calculated using the following formulae.

$$\mathrm{MCV}(\text{packed cell volume as percentage}/\text{RBC in millions}) \times 100\ \mu\mathrm{m}^3$$

$$\mathrm{MCH}(\text{Hb in grams}/\text{RBC in millions}) \times 10\,\mathrm{pg}$$

$$\mathrm{MCHC}(\text{Hb in grams}/\text{packed cell volume}) \times 100\,\text{g per 100ml}$$

For differential count, the methanol-fixed blood smears were stained by Wright–Giemsa which allows differentiation between WBC and RBC.

Statistical analysis

Results of haematological examinations were tested by the variance analysis using the Statgraphics (ANOVA–Tukey's test) software. Confidence limits set at 95 %.

Results

Growth

Different growth parameters of Caspian salmon fed diets containing Hemicell®, Natuzyme® and a combination of both, are given in Table 1. There was significant difference in FCR among fish fed the different diets ($p<0.05$). The best FCR (0.64±0.015) was in the NH group and it was significantly lower than control (0.74±0.02), N2 (0.72±0.05) and H1 (Sig.=0.035). Also the FCR in H2 (0.67±0.04) was significantly lower than H1 (0.75±0.05).

Based on our results, SGR in NH (1.01±0.01) was significantly higher than in the other groups (Sig.=0.00). N2 (0.93±0.05) was significantly higher than H2, N1, H1 and control. H2 (0.85±0.04) was significantly higher than H1 (0.82±0.01) and control (0.73±0.01).

We observed significant difference with respect to percentage body weight gain ($p<0.05$). N2 was significantly higher than N1 and H1 ($p<0.05$; Sig.=0.00). NH and N2 were significantly higher than control and H2 ($p<0.05$). We observed significant difference in CF between groups ($p<0.05$, Sig.=0.003) control and N1 was significantly lower than N2, H2 and NH.

There was significant increase in final body weight ($p<0.05$). NH was significantly higher than H1, N1, H2 and control ($p<0.05$). N2 was significantly higher than N1, H1, H2 and control ($p<0.05$).GR showed significant difference between groups ($p<0.05$; Sig.=0.00). NH and N2 showed significant increase compared to N1, H1, H2 and control ($p<0.05$). SR in N1, N2 and NH showed significant increase compared to the control group ($p<0.05$). In NH, we observed significant increase than N1.

Haematological parameters

Results of haematological parameters are shown in Table 2. No difference was observed in haematocrit%, Hb, RBC, MCV, MCH% or MCHC% ($p>0.05$). Heterophil count in control, N2, NH, H2 and H1 were significantly lower than N1 ($p<0.05$, Sig.=0.02). Lymphocytes counts in NH, H2, H1 and control was significantly higher than N1 ($p<0.05$). Lymphocyte count in H1 was significantly higher than NH ($p<0.05$). Monocyte count in NH, N1 and N2 was significantly higher than H1 ($p<0.05$). Monocyte count in N1 and N2 was significantly higher than control ($p<0.05$). Total WBC count in N2, NH and H2 was significantly higher than control, N1 and H1 ($p<0.05$). Total WBC count in N2 showed significant decrease than H2 ($p<0.05$; Table 3).

Table 1 Effect of Hemicell and Natuzyme on growth performance in *S. trutta caspius*

	NH	N2	H2	N1	H1	Control
FCR	0.64±0.01 a	0.72±0.05 b, c	0.67±0.04 a, b	0.70±0.02 a, b, c	0.75±0.05 c	0.74±0.02 b, c
SGR	1.01±0.01 e	0.93±0.05 d	0.85±0.04 c	0.82±0.01 b, c	0.78±0.04 a, b	0.73±0.01 a
BWI%	80.68±5.27 b	77.84±5.89 b	71.02±1.01 a	66.34±0.55 a	67.24±2.68 a	65.03±1.09 a
CF	1.20±0.00 c	1.19±0.00 b, c	1.20±0.00 b, c	1.18±0.00 a	1.19±0.00 a, b	1.18±0.00 a
GR (g)	0.34±0.02 b	0.33±0.02 b	0.30±0.00 a	0.28±0.20 a	0.28±0.01 a	0.27±0.00 a
Survival rate (%)	96.67±1.15 c	95.33±1.15 b, c	94.67±2.31 a, b, c	95.33±1.15 b, c	92.67±1.15 a, b	91.33±1.15 a
Final weight (g)	80.68±5.27 b	77.84±5.89 b	71.02±1.01 a	66.34±0.55 a	67.24±2.68 a	65.03±1.09 a

Means in a row with different letters are significantly different ($P<0.05$)

NH control diet+0.5 g Natuzyme®kg^{-1} and 0.5 g Hemicell® kg^{-1}, *N2* control diet+0.5 g Natuzyme®kg^{-1}, *H2* control diet+0.5 g Hemicell® kg^{-1}, *N1* control diet+0.25 g Natuzyme®kg^{-1}, *H1* control diet+0.25 g Hemicell® kg^{-1}, *Control* diet was standard trout diet and had no added multi-enzymes

Table 2 Haematological parameters in Caspian salmon (*S. trutta caspius*) at the end of feeding trial

	NH	N2	H2	N1	H1	Control
Haematocrit%	39.83±1.17	39.17±1.47	38.83±1.33	39±1.41	39±0.89	38.67±1.21
Hb (gr/dl)	8.02±0.61	7.92±0.72	7.8±0.65	7.55±0.59	7.77±0.65	8.03±0.7
WBC (N/mm^3)	7,716.67±348.8bc	7,433.30±659.3b	7,950±450.60c	6,916.67±213.70a	6,950±308.20a	6,916.67±194.10a
RBC (N/mm^3)	1.092±0.069	1.115±0.03	1.113±0.045	1.147±0.037	1.105±0.039	1.107±0.039
MCV (μm^3)	366±26.83	351.5±20.64	344.5±11.83	339.83±11.55	353.83±16.39	343.67±16.99
MCH%	73.52±4.99	70.98±5.73	69.09±7.84	65.9±5.51	70.3±5.84	72.77±7.82
MCHC%	20.14±1.6	20.22±1.81	20.12±2.02	19.41±1.96	19.91±1.52	21.21±2.46

Values are given as mean with standard error of mean (SEM). Different letters indicate significant ($P<0.05$) differences between groups

NH control diet+0.5 g Natuzyme® kg^{-1} and 0.5 g Hemicell® kg^{-1}, *N2* control diet+0.5 g Natuzyme®kg^{-1}, *H2* control diet+0.5 g Hemicell® kg^{-1}, *N1* control diet+0.25 g Natuzyme®kg^{-1}, *H1* control diet+0.25 g Hemicell® kg^{-1}, *Control* diet was standard trout diet and had no added multi-enzymes

Discussion

In the present study, fish fed the control diet exhibited lower growth and higher FCRs than diets supplemented enzymes, indicating that enzyme is beneficial for the growth of *S. trutta caspius*.

Growth response and feed utilisation were improved with enzyme supplementation, especially in NH, indicating that using these two multi-enzymes together is more effective than using them individually which is in accordance with Ghobadi et al. (2009). They supplemented rainbow trout diet with Avizyme®, enzyme containing: protease, xylanase and amylase, they showed that using multi-enzymes can increase growth parameters by decreasing negative impact of soybean in diet. Research in to the use of Natuzyme® and Hemicell® had generally concentrated on the direct application of this exogenous enzyme into plant-based poultry and pig feeds (Acamovic 2001).

Depending on the feed ingredients that were used, significant improvements in growth, feed utilisation efficiency and nutrient availability have been reported (Daşkıran and Teeter 2001). Yildirim and Turan (2010) showed exogenous enzyme (Farmazyme® containing fungal xylanase, B-gluconase, pentosonase, B-amylase, fungal B-gluconase, hemicellulase, pectinase and cellulose) in African catfish significantly improves growth performance and feed utilisation.

Boonyaratpalin et al. (2000) reported that Nile tilapia, *Oreochromis niloticus* L. fed diets with palm kernel meal (pretreated with a feed enzyme, Ronozyme VP9) showed significantly higher weight gain and apparent net protein utilisation than fish fed a similar level of untreated palm kernel meal in their diets.

Carter et al. (1994) showed that using 37.9 % soybean meal plus multi-enzyme containing carbohydranase and protease increased weight gain and decreased FCR of Atlantic salmon, in comparison with diet containing fish meal.

According to Rodehutscord and Pfeffer (1995), Phytase in Natozyme® showed potential effects on digestibility and utilisation of phosphorus originating from plant materials; and the addition of phytase in plant diet can increases weight gain in rainbow trout. Cao et al. (2007) showed that the indigestible phytate phosphorus was successfully converted to available phosphorus by phytase which mean improvement in phosphorus utilisation.

Karimi et al. (2009) observed the decreasing effects of 0.05 % multiple enzyme mixture with canola meal on FCR and they showed improvement in SGR of rainbow trout and that it can also be cost effective.

Table 3 Leukocyte profile of Caspian salmon (*S. trutta caspius*) at the end of feeding trial

	NH	N2	H2	N1	H1	Control
Heterophil (N/mm^3)	10±0.89	9.67±1.63	10.17±0.75	11.67±1.03	9.5±1.05	10±1.27
Lymphocyte (N/mm^3)	87.67±0.52 b,c	87.33±2.07 a, b, c, d	88.00±0.89 b, c, d	85.5±1.05 a	89.00±0.89 d	88.33±1.03 c, d
Monocyte (N/mm^3)	1.83±0.75 b, c	2.33±0.82 c	1.17±1.17 a, b, c	2.17±0.75 c	0.50±0.55 a	1.00±0.63 a, b
Eosinophil (N/mm^3)	0.67±0.52	0.67±0.52	0.67±0.52	0.67±0.52	1.00±0.00	0.67±0.52

Values are given as mean with standard error of mean (SEM). Different letters indicate significant ($P<0.05$) differences between groups

NH control diet+0.5 g Natuzyme® kg^{-1} and 0.5 g Hemicell® kg^{-1}, *N2* control diet+0.5 g Natuzyme®kg^{-1}, *H2* control diet+0.5 g Hemicell® kg^{-1}, *N1* control diet+0.25 g Natuzyme®kg^{-1}, *H1* control diet+0.25 g Hemicell® kg^{-1}, *Control* diet was standard trout diet and had no added multi-enzymes

The results of the current study showed that optimum growth performance and feed efficiency were obtained at NH enzyme complex-supplemented diet. This result is in agreement with previous studies on channel catfish (Jackson et al. 1996), *P. pangasius* (Debnath et al. 2005), *C. batrachus* × *C. gariepinus* (Giri et al. 2003), *O. niloticus* × *O. aureus* (Lin et al. 2007) and rainbow trout (Farhangi and Carter 2007). A significant body weight improvement was also reported in turkeys and poultry by Leeson et al. (1996) and Ghazi et al. (2003) in diet supplemented with exogenous enzymes (containing xylanase).

Dietary enzymes can be used to supplement the fish's own enzyme production including amylases to improve starch digestibility, proteases to improve protein digestibility and lipases to improve lipid digestibility (Lin et al. 2007; Zhou et al. 2009). The results of Van Weerd et al. (1999) and Ng and Chen (2002) indicated that exogenous enzyme supplementation can promote the secretion of endogenous enzymes. Abo-State et al. (2009) suggested that the supplementation of phytase (75 and 150 mg/kg) in DDGS-based diets can significantly improve growth and feed utilisation parameters in Nile tilapia (*O. niloticus*) fingerlings.

Recent research with broilers and fish suggest that not only are the types of enzymes critical but also their concentration (relative to body weight) and the direction (positive or negative) of an animal's response to supplementation (Bedford and Inborr 1993; Lin et al. 2007).

Thus, the use of H2 and NH led to the highest WBC count, indicating that multi-enzymes including β-mannans, xylanase, in Hemicell and Natozyme crossing the intestinal mucosa are potent stimulators of the innate immune system, resulting in increased proliferation of macrophages and monocytes and resultant cytokine production (Ehsani and Torki 2010).

In conclusion, enzyme concentration plays an important role in diets; addition of multi-enzyme complexes (Natuzyme® and Hemicell®) resulted in significant improvements in body weight gain and feed efficiency in *S. trutta caspius*. On the other hand, effects of the enzyme supplement on intestinal microbial flora and improvement in growth by the release of a growth-enhancing factor cannot be neglected. Further investigations can be carried out to focus on the substrates themselves in combination with management practices, which may also affect the results.

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