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The Effects of Dietary Supplement of Immunogen on Growth Performance, and Visceral and Hepatic Somatic Indices of Juvenile Rainbow Trout, *Oncorhynchus mykiss* (Walbaum 1792)

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Abstract

Prebiotics have been introduced as non-digestible feed ingredients, which stimulate growth and activity of beneficial bacteria. This study was conducted to evaluate the effects of Immunogen, a mixture of prebiotic on the growth performance and visceral and hepatic somatic indices in rainbow trout, *Oncorhynchus mykiss* (Walbaum 1792). A basal diet was formulated using common feed ingredients supplemented with Immunogen at 0, 1, 1.5, and 2 $g \cdot kg^{-1}$, leading to four experimental diets. Each experimental diet was randomly assigned to triplicate 1,500 L tanks. Rainbow trout with an initial weight of 99.59±0.74 g were randomly distributed in the experimental tanks. Results showed that inclusion of dietary Immunogen significantly increased the final weight and specific growth rate (SGR) ($p<0.01$) of rainbow trout compared to the control. Feed conversion ratio (FCR) and protein efficiency ratio (PER) were also improved after prebiotic administration to the experimental fish ($p<0.01$ and < 0.05, respectively). Dietary addition of 2 $g \cdot kg^{-1}$ prebiotic also increased the relative liver and visceral weights ($p < 0.05$). The body composition and blood cortisol level were not influenced by the prebiotic inclusion ($p>0.05$). In conclusion, administration of the prebiotic Immunogen is capable of improving the nutrient efficiency and growth performance of rainbow trout confirming the positive effect of a mixture of prebiotics on the fish.

Introduction

In commercial farms, unfavourable environmental conditions and/or poor management practices may cause stress (Mazur and Iwama 1993; Amirkolaie 2011), leading to growth reduction and suppression of immune system in fish. In this condition, fish will be more susceptible to disease outbreaks (Gibson 1998). Traditionally, antibiotics have been introduced as a solution to fight

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disease or to enhance growth performance in fish (Klaenhammer and Kullen 1999; Hoseinifar et al. 2010). However, increasing use of antibiotics may have negative side effects such as the development of antibiotic-resistant bacteria rendering the use of antibiotics as growth promoters undesirable (Boyd and Massaaut 1999; Esiobu et al. 2002; Cabello 2006).

Social and economic concerns about the increasing use of antibiotics and other chemicals in fish farming have encouraged more environmentally friendly approaches for increasing growth (Verschuere et al. 2000; Ebrahimi et al. 2013). Therefore, alternative techniques are needed to replace the use of drugs and in this regard, the contribution of prebiotics may be significant. Prebiotics have been defined as non-digestible food ingredients that benefit the host by selective stimulation of the growth and/or the activity of health-promoting bacteria in the gastro-intestinal tract (Gibson et al. 2004; Merrifield et al. 2010; Dimitroglou et al. 2011).

A number of studies have demonstrated that the administration of prebiotics can improve the growth performance, disease resistance and gut morphology, and also modulate the gut micro-organisms in various fish species (Stoykov et al. 2007; Burr et al. 2008; Salze et al. 2008; Dimitroglou et al. 2009). Dietary supplementation of the polysaccharide prebiotic inulin has been shown to enhance growth rate of Siberian sturgeon, *Acipenser aerie* Brandt 1869 (Mahouts and Olivier 2005), and African catfish, *Clarias gariepinus* (Burchell 1822*)* (Mahious and Ollevier 2005). Feed efficiency and survival rate of juvenile red drum, *Sciaenops ocellatus* (Linnaeus, 1766) were improved by dietary prebiotics such as mannan-oligosaccharides, trans-galacto-oligosaccharides, and GroBiotic (Buentello et al. 2010). Immunogen (a commercial prebiotic) contains various stimulating components such as mannan-oligosaccharide and β-glucans, which have been used as feed additives in various animals. Supplementation of Immunogen improved performance and disease resistance of common carp, *Cyprinus carpio* Linnaeus 1758, (Ebrahimi et al., 2013) and reproductive performance of platy, *Xiphophorus maculatus* (Gűnther 1866) (Hajibeglou and Sudagar 2011).

Factors associated with fish farming production such as culture density, handling and transport may cause stress thus influencing the homeostatic system and the physiological state of the fish (Pages et al.1995; Waring et al. 1996; Martins et al. 2006). Manipulation of fish intestinal microbiota by dietary supplementation of prebiotics may enhance fish tolerance to environmental stress through enhanced non-specific immune responses (Bailey et al. 1991).

While some literature is available on the effects of different types of prebiotics on fish performance and health related parameters, there is little information available on the effect of prebiotic on the morphometrics of commercial fish. Supplementation of mannan-oligosaccharides changed intestinal morphology in rainbow trout and white seabream *Diplodus sargus* (Linnaeus 1758) (Yilmaz et al. 2007; Dimitroglou et al. 2009). In addition, previous studies on prebiotic mostly focused on different types of oligosaccharides alone as a source of prebiotic, but little is known about the efficiency of a mixture of carbohydrate sources. Therefore, the main goal of this

study was to assess the effects of Immunogen as a mixture of prebiotic on the performance and morphometrics (visceral and hepatic somatic indices) in rainbow trout.

Materials and Methods

Experimental system and animal

This study was carried out at the experimental facility of Shahid Rajaei fish farm located in the northern part of Iran. In this study, rainbow trout juveniles, bred at a nearby reproduction facility, were adapted to a commercial diet and the new environment for a week before the start of the experiment. After adaptation, the fish were divided randomly into 12 tanks of 1,500 L capacity, with an initial stocking density of 50 individuals (average weight of 99.59±0.74 g) per tank. The experiment lasted for 8 weeks. The prebiotic Immunogen used in this study was composed of mannan-oligosaccharide 18%, β-glucans (1-3, 1-6) 30%, protein 33%, ash 9%, moisture 8%, and fibre 2% (Provided by Soroush Radian Co., Tehran, Iran).

A basal diet was formulated using locally grown feed ingredients with estimated gross energy and protein levels of 12.56 $MJ \cdot kg^{-1}$ and 433$g \cdot kg^{-1}$, respectively (Table 1). Four incremental levels (0, 1, 1.5, and 2 $g \cdot kg^{-1}$ prebiotic diet) of Immunogen were added to the basal diet to prepare four experimental diets. All ingredients were finely ground, mixed and pelletized. A pellet maker with 4 mm diameter die was used for producing the diets. All four diets were air-dried at 50 ^{0}C at the same time and stored at -20 ^{0}C until use.

Table 1. The percentage of ingredient used in the experimental diet on % dry matter weight basis.

Experimental diet	Amount
Soybean meal	15
Fish meal	35
Meat meal	22.5
Corn	15.82
Wheat	2
Fish oil	5
Molasses	2
DL-methionine	0.84
Lysine	0.84
[1]Premix	1

Nutrient composition of the experimental diet in g/kg

Dry matter	938.0
Crude protein	433.2
Crude fat	111.3
Carbohydrate	254.9
Crude ash	133.6

[1]premix consisted of equal proportion of vitamins and minerals
Vitamin premix consisted of (g/kg premix):1200000 IU Vitamin A, 400000 IU Vitamin D3, 3000 IU Vitamin E,1200 mg Vitamin K3, 5400 mg Vitamin C, 200 mg Vitamin B1, 3360 mg Vitamin B2, 7200 mg Vitamin B3, 9000 mg Vitamin B5, 2400 mg Vitamin B6, 600 mg Vitamin B9, 4 mg Vitamin B12, 500mg Antioxidant, up to 1kg carrier.

Mineral premix consisted of (g/kg premix): 2600 mg Mn, 600 mg Cu, 6000mg Fe, 4600mg Zn, 50mg Se, 100mg Iu, 50 mg Co, 100000 mg choline chloride, up to 1 kg carrier (composed of wheat bran).

Experimental procedure

Fish were weighed on the 1st and the last day of the experiment. Fish were hand fed the experimental diets at the rate of 1.4-1.8% of biomass, three times per day (8.00, 13.00, and 18.00 h). Experimental diets were randomly assigned to one of 12 tanks, having three replicates per diet. Water quality parameters were monitored daily to ensure they were in appropriate range for the fish. The water temperature and pH ranged between 14-16 ^{0}C and 7.3-7.9, respectively, during the experiment. Water flow which was checked daily for each tank was 12 L·min^{-1}.

On day 56, all fish were weighed. Beforehand, five fish were randomly selected from each tank, sacrificed using an overdose of clove essence solution and used for analysis of body composition. In addition, three fish were randomly selected from each tank for liver and visceral morphometric analyses. The whole viscera were removed and livers were separated from the viscera. The weight of the viscera was recorded. Blood samples were also collected for plasma cortisol analysis. For this purpose, three fish were randomly selected from each tank before weighing and immediately anaesthetized using clove essence solution. One mL of blood was collected by caudal vein puncture using syringes containing 3 mg of Na_2EDTA. The collected samples were placed in cooled, 1.5 mL plastic tubes, then mixed, and centrifuged at 4,000 ×g for 10 min at 4 ^{0}C. After centrifugation, plasma was collected and stored at -20 ^{0}C for the cortisol analyses.

Chemical analysis

Feed samples were collected and pooled at regular intervals during the experimental period and ground using a 1mm screen before analyses. Feed and fish body were analysed for dry matter by drying samples for 24 h at 103 ^{0}C until constant weight was obtained (ISO 6496 1983). Ash content was determined by incineration in a muffle furnace for 4 h at 550 ^{0}C (ISO 5984 1978). Crude protein (N×6.25) was measured by the Kjeldahl method after acid digestion according to ISO 5983 (1979). Lipid was extracted with petroleum ether in a Soxhlet apparatus. Carbohydrate fraction was determined as dry matter minus fat, protein, and ash in the feed. Plasma cortisol levels were measured by radio immunoassay (RIA) according to Rottlant et al. (2001) and expressed as ng·mL^{-1}.

Fish performance

Weight gain was determined by the difference between initial and final body weights. Feed conversion ratio (FCR) was calculated per tank from feed intake data and weight gains: FCR = feed consumed (g) / wet body weight gain (g). Specific growth rate (SGR) was calculated as follows and expressed as a percentage: SGR = 100 (Ln W_{final}- Ln $W_{initial}$) × days^{-1}. The calculations were based on the dry weight of the diets. Protein efficiency ratio (PER) was calculated per tank from weight gain data and diet crude protein as follows: PER = weight gain (g)/ protein intake (g).

Hepatic and visceral somatic indices were calculated according to the following formulas:

Hepatic somatic index (HSI%) = 100 × (liver weight/whole bodyweight)

Visceral somatic index (VSI%) = 100 × (visceral weight/whole bodyweight)

Statistical analysis

Data are presented as means of each treatment ± standard deviation. All data were checked for normality after transformation (ASIN). Homogeneity of variances was tested using Levene's *F* test. One-way ANOVA was used to determine the effects of Immunogen levels on fish performance and cortisol level. The means were compared by a Tukey's post hoc test. For all statistical analyses, each tank was considered as the experimental unit.

Results

The inclusion of different levels of prebiotic influenced growth related parameters during the 8-week experiment (Table2) Rainbow trout significantly gained more weight with administration of all the tested levels of Immunogen ($p<0.05$). Along with the growth, FCR and SGR were also significantly improved in rainbow trout fed the prebiotic diets in comparison to control diet ($p<0.05$). However, growth related parameters were similar in fish fed the three different prebiotic levels. Similar to the growth parameters, PER increased in rainbow trout fed the prebiotic diets ($p<0.05$).

Table 2. Growth performance in rainbow trout feeding on different levels of Immunogen (IMO $g \cdot kg^{-1}$) over 56 days experimental period. All values are means ±standard deviation of triplicate tanks/treatment.

	Diet			
Growth Parameters	Control	IMO 1	IMO1.5	IMO 2
Initial weight (g)	100.2±1.3	99.0±2.1	99.8±2.3	100.3± 1.5
Final weight (g)	220.4 ± 0.8^{b}	269.6 ± 3.6^{a}	270.8 ± 2.3^{a}	268.9 ± 4.2^{a}
Biomass gain (g)	120.2 ± 2.78^{b}	170.4 ± 2.63^{a}	170.0 ± 3.13^{a}	168.6 ± 2.26^{a}
SGR (%)	1.20 ± 0.16^{b}	1.36 ± 0.13^{a}	1.35 ± 0.06^{a}	1.36 ± 0.03^{a}
FCR	1.41 ± 0.16^{b}	1.13 ± 0.09^{a}	1.11 ± 0.06^{a}	1.13 ± 0.14^{a}
PER	1.41 ± 0.32^{b}	1.82 ± 0.26^{a}	1.80 ± 0.15^{a}	1.80 ± 0.27^{a}

Different superscript letters in each row show significant differences

VSI and HSI were also affected by the prebiotic. Both VSI and HSI were larger in rainbow trout fed on the diet containing 2 $g \cdot kg^{-1}$ Immunogen in comparison with the other diets ($p<0.05$; Table 3). Moreover, administration of 1.5 $g \cdot kg^{-1}$Immunogen led to larger VSI values ($p<0.05$) whereas it did not affect HSI of rainbow trout.

The body composition results demonstrated that the inclusion of dietary prebiotic did not affect the carcass composition of rainbow trout (Table 4). Plasma analysis also revealed that cortisol concentrations were not significantly influenced by administration of different doses of Immunogen ($p> 0.05$; Fig. 1). However, there was a trend towards larger concentrations of cortisol with Immunogen diets.

Table 3. Organ characteristics of rainbow trout fed on different levels of Immunogen (IMO $g \cdot kg^{-1}$) over 56 days experimental period. All values are means ± standard deviation of triplicate tanks/treatment.

	Diet			
Organ characteristics	Control	IMO 1	IMO 1.5	IMO 2
HSI (%)	2.3 ± 0.3^{b}	2.2 ± 0.01^{b}	2.7 ± 0.8^{b}	3.15 ± 0.25^{a}
VSI (%)	13.2 ± 0.07^{c}	13.0 ± 0.09^{c}	14.1 ± 0.2^{b}	15.2 ± 0.35^{a}

Different superscript letters in the same row show significant differences

Table 4. Body composition of rainbow trout fed on different levels of Immunogen (IMO $g \cdot kg^{-1}$) over 56 days experimental period. All values are means ± standard deviation of triplicates tanks/treatment.

		Diets			
Parameters	Initial	Control	IMO 1	IMO 1.5	IMO 2
Dry mater	24.6	25.64±0.32	25.61±1.01	25.66±2.77	25.77±1.24
Protein	12.43	14.88±1.22	15.00±1.46	15.16±0.91	14.93±0.09
Fat	5.80	6.98±0.18	7.08±0.28	7.02±0.96	7.05±0.26
Ash	3.80	3.08±0.23	2.98±0.16	2.75±0.13	3.15±0.21

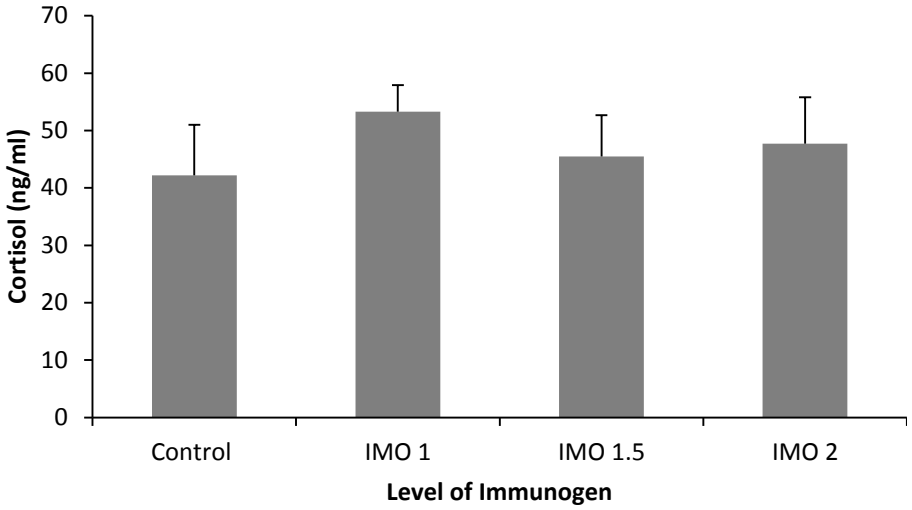

Fig. 1. Plasma cortisol levels in rainbow trout after being fed for 56 days on different concentrations of Immunogen (IMO). All values are means ±standard deviation of triplicate tanks/treatment.

Discussion

The present study showed that supplementation of prebiotic Immunogen improved growth related factors (final weight, biomass gain, SGR and FCR) in rainbow trout. A large number of previous studies also showed positive effects of different dietary prebiotics on fish performance, feed efficiency and immune response of aquatic animals (Staykov et al. 2007; Torrecillas et al.2007; Burr et al. 2008; Taati et al. 2011).The beneficial effect of prebiotic on growth may be related to its effect on the microbial flora of the intestine. Prebiotics act as media and induce colonization of useful bacteria such as *Lactobacillus* (Mahious et al. 2006; Jafarnodeh 2010). Lactic acid bacteria inhibit specific fish pathogens by production of bacteriocins (Thompson et al.1999; Ebrahimi et al. 2013). They also have the ability to secrete a wide range of exo-enzymes (Moriarty 1996, 1998; Suzer et al. 2008) and increase digestive enzyme activity (Askarian et al. 2011) thus leading to a better nutrient digestion thereby achieving a better growth.

Along with the growth parameters, larger PER values in rainbow trout fed prebiotic diet may be also attributed to a better protein utilization induced by the prebiotic. Nutrients utilization might have been improved at similar ratios and this probably masked the effect of PER on carcass protein content. Meanwhile, a nitrogen balance for rainbow trout fed different levels of Immunogen is necessary to determine clearly why changes in PER did not lead to changes in carcass protein content.

Although the gut morphology was not tested, a better growth performance of rainbow trout may be also caused by changes in the micro-villi morphology and density induced by the dietary prebiotic.

There is evidence showing a possible effect of dietary prebiotic on gut morphology. Supplementation of 0.2% dietary mannan-oligosaccharide increased intestinal micro-villi length of cobia larvae, *Rachycentron canadum* (Linnaeus 1766) (Salze et al. 2008). Dimitroglou et al. (2010) also observed increased densities of micro-villi in both the anterior and posterior intestinal regions of gilthead sea bream, *Sparus aurata* Linnaeus 1758, by supplementation of dietary mannan-oligosaccharides. We hypothesize that administration of the prebiotic may have resulted in elongated intestinal micro-villi and increased density leading to an improved nutrient absorption.

A larger VSI observed in rainbow trout fed the prebiotic diets may be due to an increase in villi height and density. Prebiotic inclusion also had an impact on the intestinal weight. Refstie et al. (2006) observed heavier intestines in fish fed prebiotic diets in comparison to soybean or the control. In the current study, Immunogen supplementation (2 gkg^{-1}) also increased liver weight. Similarly, supplementation of prebiotic increased liver weight in *Salmo salar* Linnaeus 1758 (Refstie et al. 2006). This result may suggest that growth promotion induced by prebiotic is associated with larger liver fat deposition in rainbow trout. However, further measurement of intestine morphology and liver composition will be necessary to assess better the impact of prebiotic on the organs' health.

We believed that prebiotic administration may have resulted in lower stress levels in fish, but the current results showed no effect of prebiotic on the stress level of rainbow trout as there was no significant change in the cortisol levels. It should be noted that in the current study all water quality parameters were kept within the acceptable range for rainbow trout and did not stress the fish. However, a stress test at the end of the study would enable a better understanding of the effect of prebiotic on the stress response in fish.

Conclusion

The results of the current study demonstrate that the administration of Immunogen as a dietary supplement can improve growth performance and protein efficiency in rainbow trout. The effect of Immunogen on the characteristics of liver and viscera may indicate that growth improvement induced by the prebiotic may be associated with morphological changes in the organs. The positive impact of prebiotic on stress alleviation in fish is not proven yet. Further stress tests are needed to gain more insights into the relationship between stress response and prebiotic administration in fish.

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