Growth yield, survival, carcass quality, haematological, biochemical parameters and innate immune responses in the grey mullet (*Mugil cephalus* Linneaus, 1758) fingerling induced by Immunogen® prebiotic

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ABSTRACT

The present study goals to shed lights on the effect of prebiotic Immunogen on growth performance, survival, carcass quality, haematological and biochemical factors as well as innate immune responses of the grey mullet (*Mugil cephalus* L.). Basal diets as prebiotics at three concentrations were added to grey mullet: Treatment-1, 0.5 g kg\(^{-1}\); Treatment-2, 1 g kg\(^{-1}\) and Treatment-3, 2 g kg\(^{-1}\). Number of 12 fiberglass tanks (60-L) with 3 replicates for the treatment group (n = 30 per tank with average initial weights 8.32 ± 0.39 g) and the control group was considered. After 60 days, grey mullet receiving the experimental diets showed a significantly better growth yield compared to those fed the control diet. Addition of prebiotic to experimental diets led to significant difference (p < .05) in the glucose and WBC while this was not case for haemoglobin, haematocrit, MCV, MCH, MCHC, RBC, lymphocyte, neutrophil, eosinophil, albumin, globulin and total protein (p > .05). At the end, humeral innate immune responses (Ig levels and lysozyme activity) were significantly higher in 2 g kg\(^{-1}\) immunogen-fed fish (p < .05). The results indicate that the application of prebiotic to the diet of grey mullet showed a positive effect on growth, glucose, WBC rates and innate immune responses.

1. Introduction

In aquaculture, the use of antibiotics has been limited due to development of antibiotics-resistant bacteria strains and changes in the immune response. Recently, prebiotics as non-digestible forage additives stimulate function or abundance of useful gastrointestinal bacteria and have received much more interest, thanks to enhanced production, health and disease resistance of aquatic animals among many others (Dimitroglou et al. 2011). Prebiotics are carbohydrates which can be classified according to molecular size or polymerization rate, including monosaccharides, polysaccharides or oligosaccharides. As the International Union of Pure and Applied Chemistry nomenclature implies, oligosaccharides are defined as saccharides containing about 3–10 sugar moieties (Mussatto & Mancilha 2007).

Prebiotics have been incorporated with some carbohydrates – including mannan oligosaccharides (MOSs), oligofructose and inulin (Teitelbaum & Walker 2002; White et al. 2002) – and compounds such as peptides, proteins and some among many others (Gibson 1998; Fooks et al. 1999). Some prebiotics have shown positive effects on the growth yield, haematological and serum biochemical parameters, innate immunity, microbial fermentation and autochthonous intestinal microbiota population of some species (Jeney & Jeney 2002; Mahious et al. 2006; Mohajer Esterabadi et al. 2010; Hoseinifar, Mirvaghefi, Merrifield, et al. 2011; Hoseinifar, Mirvaghefi, Mojazi Amiri, et al. 2011; Ta’ati et al. 2011).

Here, a commercial prebiotic, Immunogen, was considered. Foregoing prebiotic is found to be a natural product characterized with various triggering compounds, including b-glucan and MOSs, used as feed additives in enormous animals. MOSs can be perceived as glucomannoprotein complexes obtained from a yeast cell wall (*Saccharomyces cerevisiae*), and b-glucans act as complex carbohydrates which are derived from yeasts and fungi (Sohn et al. 2000). Incorporation of MOS in fish diets is proved to be useful to enhance health condition and growth yield in fish (Staykov et al. 2007; Torrecillas et al. 2007; Soleimani et al. 2015) and at the same time it enhances the innate immune responses as well as many other advantages (Soleimani et al. 2015). At the same time, other prebiotic (including inulin) has positive impacts on intrinsic immune response in some fish and shellfish species (Mahious et al. 2006; Mohajer Esterabadi et al. 2010). However, there is a lack of literature information on prebiotic effects on marine fish. *Mugil cephalus* has a promising market potential in Europe, East and South Asia. At the same time, it is an important aquaculture species in Iran. The consumers demand has accelerated the aquaculture development of these species in Asian countries (Yelghi et al. 2012). Many authors have dealt with the main haematological and biochemical parameters in this fish species under different conditions (Fazio et al. 2013; Fazio, Cecchini, et al. 2014; Fazio, Faggio, et al. 2014; Fazio et al. 2015); however, no studies were carried out on the effect of Immunogen as prebiotic on haematology and biochemistry of mullets. Hence, in the present research, we aimed to assess the effects of several
levels of the prebiotic Immunogen on growth yield, survival, carcass quality, haematological and biochemical parameters, and innate immune responses of mullet fingerlings.

2. Materials and methods

2.1. Fish

A total number of 360 grey mullets (mean initial weight of 8.32 ± 0.39 g) (mean ± SD) at mid-February 2014 were captured from the coastal water of Chabahar for quarantine and health check purposes. Fish were acclimatized for one week in 400-L tank and then were fed with commercial diet. Water was exchanged (50%) in daily manner and throughout experiment water quality was monitored weekly. Salinity, temperature, dissolved oxygen concentration, ammonia nitrogen concentration and pH were measured about 38 g L⁻¹, 28.2°C ± 0.5, 7.01 ± 0.87 mg L⁻¹, 0.11 ± 0.04 mg L⁻¹ and 7.8 ± 0.4, respectively. Ad labium with commercially available pelleted feeds were fed to Fish (Beyza Feed Mill Company, Iran) at the rate of 3% of body weight followed by biomass assessment by bulk weighting every 7 days (n = 30 for each group). The daily ration was subdivided into two parts and fed at 9:00 hours and 16:00 hours for 8 weeks to fish.

2.2. Experimental design and feeding diet

A basic diet was dedicated to grey mullet fingerling (Table 1); the foregoing basal diet was considered as control diet and to prepare experimental diets, basal formulation was supplemented with variable levels of Immunogen (at 0, 0.5, 1 and 2 g kg⁻¹). Prebiotic, Immunogen was provided by Soroush Radian Co, Tehran, Iran. 1.0-mm sieve was used to homogenize feedstuffs, mixed in the computed ratios and soaked with dis-tilled water (25%, v/w) to be pelleted in a grinder. Diets were

Table 1. Dietary formulations (%) and proximate composition.

| Nutrient material       | Control | Treatment1 | Treatment2 | Treatment3 |
|-------------------------|---------|------------|------------|------------|
| Fish meal               | 60      | 60         | 60         | 60         |
| Flour wheat             | 0.04    | 0.04       | 0.04       | 0.04       |
| Gain wheat              | 0.04    | 0.04       | 0.04       | 0.04       |
| Corn wheat              | 0.03    | 0.03       | 0.03       | 0.03       |
| Soybean wheat           | 9       | 9          | 9          | 9          |
| Fish oil                | 0.05    | 0.05       | 0.05       | 0.05       |
| Soybean oil             | 3       | 3          | 3          | 3          |
| Colza oil               | 2       | 2          | 2          | 2          |
| Lecithin                | 2       | 2          | 2          | 2          |
| Antioxidant             | 0.30    | 0.30       | 0.30       | 0.30       |
| Vitamins and minerals*  | 2       | 2          | 2          | 2          |
| Dicalcium phosphate     | 0.70    | 0.70       | 0.70       | 0.70       |
| Prebiotic Immunogen     | 0       | 0.05       | 0.1        | 0.2        |
| Chemical composition (% or cal. g⁻¹) |
| Protein (%)             | 0.5 ± 40.1 | 0.2 ± 40.18 | 0.4 ± 40.28 | 0.3 ± 40.2 |
| Lipid (%)               | 0.3 ± 18.8 | 0.2 ± 18.25 | 0.4 ± 18.17 | 0.3 ± 18.19 |
| Moisture (%)            | 0.1 ± 8.73 | 0.3 ± 8.65  | 0.2 ± 8.58  | 0.4 ± 8.68  |
| Ash (%)                 | 0.06 ± 9.8 | 0.07 ± 9.9  | 0.05 ± 9.6  | 0.07 ± 9.7  |
| Energy crude (cal. g⁻¹) | 71 ± 4980 | 54 ± 4985  | 41 ± 4975  | 50 ± 4978  |

Notes: Control group was fed with basal diet and the remaining treatments (1, 2 and 3) were fed with 0.5, 1 and 2 g kg⁻¹ prebiotic in diets, respectively.

*Vitamins and minerals were supplied according to NRC.

The present research was done over a period of 60 days to assess prebiotics efficiency on growth yield, survival, carcass quality, haematological, biochemical parameters and innate immune responses of the grey mullet (M. cephalus). Grey mullet fingerlings (n = 360) were classified into four classes. The number of three replicates was used in each group and randomly assigned to 12 plastic tanks each characterized with volume of 60 L. Basal diet was fed to the control group (1), and the remaining groups (2, 3 and 4) were fed with 0.5, 1 and 2 g kg⁻¹ prebiotic in diets, respectively.

2.3. Sample collection and analysis

At the end of experiment, all fish were individually weighted to ensure a homogenous sampling. Clove powder was used to anesthetize fish (n = 60 for each treatment) (5 mg L⁻¹), the specimens were individually weighted and measured to estimate final weight, final length, body weight increase, specific growth rate (SG), condition factor (CF), feed conversion rate (FCR), protein efficiency ratio (PER) and hepatic somatic index (HSI). At the same time, the number of nine fish (three fish per tank) from each treatment was selected at equivalent weight to analyse whole body proximate composition. Standard procedure of Association of Official Analytical Chemists (AOAC 1995a, 1995b) was used for chemical analysis of fish whole body to determine moisture content oven drying at 105°C for 10 h (constant weight) was considered. As per the Kjeldahl method, crude protein was measured by analysis of total nitrogen (CP = N × 6.25) in an indirect manner. Crude lipid was determined using Soxhlet apparatus, weighting samples in a porcelain crucible placed in furnace at 550°C for 24 h (AOAC 1995a, 1995b) to measure ashes. At the same time, the number of three fish of each tank was dissected, and hepatics were mixed and weighted to determine the HSI (=100 × (hepatic weight/total body weight). Whole-body SG rates, which is expressed as a percentage of the body weight, were calculated using the growth rate equation of SG (%/day)=([ln(Wf) − ln(Wi)] × 100)/t, where Wi and Wf are the
early and final wet weights (g) of the experimental grey mullet, respectively, and \( t \) is the length of the experimental period (in days). The feed conversion ratio (FCR) was calculated in respect to wet weight as: FCR = wet weight of feed consumed/change in wet weight. Total feed consumption was estimated from the amount of not eaten feed and was collected from the strainer at the bottom of the tank every hour. The pellets were remained intact before collection and unused feed was calculated from pellets using the average weight of a pellet for each feed. The PER and per cent of survival rate was calculated in terms of wet weight as: PER = Live weight gain (g)/Protein intake (g); Survival (%) = Total live fish (No.) after \( t \)/Total fish at 0 day (No.) × 100.

2.4. Haematological and biochemical analyses

The number of nine fish from each treatment was anesthetized (with clove oil at 5 mg L\(^{-1}\)) and blood samples were taken after excising caudal peduncle and were transferred to un-heparinized and heparinized sterile tubes 1–1.5 mL for the haematological and serum biochemical tests proposes. Serum was removed from the clotted sample after centrifugation at 2795 g for 5 min and frozen at 80°C until analysis (Shaluei et al. 2012). The number of white blood cell and red blood cell tests was determined soon on fresh blood. The number of blood leukocytes and erythrocytes was counted by diluting heparinized blood with Giemsa stain at 1:30 dilution, and the cells were counted using a haemocytometer Neubauer under the light microscope (Stevens 1997).

The leucocyte differential count was made in peripheral blood smears stained by Merck Giemsa (Svobodova et al. 1991; Jahanbakhshi et al. 2012), resulting in the neutrophil value of differential neutrophils and the mononuclear value of differential lymphocytes as well as monocyte and eosinophil. Haematocrit values (Ht, %) were soon measured after sampling through putting fresh blood in glass capillary tubes and were centrifuged for 5 min at 10,000 rpm in a microhaematocrit centrifuge (Hettich, Germany) and then packed cell volume was measured (Goldenfarb et al. 1971). Haemoglobin levels (Hb, mg/L) were determined colorimetrically by measuring the formation of cyano-methemoglobin according to Lee et al. (1998).

Erythrocytes parameters (MCHC, MCV and MCH) were estimated from red blood cell (RBC), Ht and Hb, as described by Lee et al. (1998). The method developed by Burtis and Ashwood, (1994) was used to measure albumin. Globulin was calculated through subtracting albumin level from total protein. Albumin value was divided by globulin value to calculate albumin/globulin ratio. Plasma glucose was quantitatively measured using commercially available diagnostic Experimental Protocols kits Pars Azmoon, Iran, at 546 nm and 37°C by the glucose oxidase method (Hedayati et al. 2008). Glucose was measured by spectrophotometric method (WPAS2000-UV/VIS, Cambridge, UK) with reagents provided in standard analyses kits (Pars Azmon, Iran) (Shaluei et al. 2012). Spectrophotometry was used to determine plasma cholesterol and plasma total protein levels as per commercial kits Sigma 337-B and Sigma 401-25P by approach developed by Canli (1996).

2.5. Immunological assays

According to the method described by Siwicki and Anderson (1993), serum total immunoglobulin (Ig) levels were determined. Shortly, the microprotein determination method was used to shed lights on serum total protein content (C-690; Sigma), before and after precipitating immunoglobulin molecules, using a 12% solution of polyethylene glycol (Sigma). The difference in protein content denotes to Ig content. Serum lysozyme activity was determined by the method described by Demers and Bayne (1997) and lysis of the lysozyme-sensitive Gram-positive bacterium Micrococcus lysodeikticus (Sigma) was considered.

2.6. Statistical analysis

All data were analysed by SPSS 16.0 for windows. One-way analysis of variance was used to determine whether significant variation existed between fish fed the experimental diets for 60 days. Difference between means were compared using the Duncan’s test with 5% (\( p < .05 \)) significance. Normality was tested using the Kolmogorov–Smirnov test. Leven’s test was carried out to verify the homogeneity of variance. Non-homogenous data were arcsine transformed before further statistical analysis. Data were presented as means ± SEM of three replications.

3. Results and discussion

3.1. Survival and growth

Data on growth yield of fish fed in conjunction with four different diets can be seen in Table 2. Weight gain, SG rate, FCR, CF, HSI and PER were significantly differed (\( p < .05 \)) in fish fed with experimental diet.

3.2. Carcass composition

The level or type of alternative ingredient in diets did not affect protein, lipid, ash and moisture contents of fish (Table 3). Plasma glucose, cholesterol, albumin, globulin and total protein values can be seen in Table 4. Plasma glucose concentrations increased in three treatments under increased prebiotic Immunogen in experimental diets caused that. The highest glucose concentrations were observed in fish fed 2 g kg\(^{-1}\) and the lowest was found in fish fed 0 g kg\(^{-1}\) prebiotic Immunogen, respectively.

Haematological parameters did not differed significantly including, haematocrit, MCH, MCHC, MCV, RBC, lymphocyte, neutrophil, and eosinophil plasma as it can be seen in (Table 5). Blood WBC percentage was increased in one, two, three treatments, respectively, as prebiotic Immunogen in experimental diets was increased. The highest and lowest WBC percentages were found in fish fed 2 g kg\(^{-1}\) and those with 0 g kg\(^{-1}\) prebiotic Immunogen, respectively (Table 5).

3.3. Innate immune responses

Figures 1 and 2 show impacts of the prebiotic on the humoral innate immune responses of mullet. Both innate immune
responses measured were significantly higher \((p < .05)\) in one, two and three treatments fed fish compared to the control one. Fish fed 2 g kg\(^{-1}\) prebiotic had significantly increased lysozyme and Ig activity than the control group.

A wide variety of useful feed additives, including probiotics and prebiotics with useful effects to the host, were exploited in aquaculture to combat diseases (including supplements), to enhance growth (including increasing the size and weight gain) and, in some cases, to act as an alternative antimicrobial compounds (Irianto & Austin 2002) and to stimulate immunity response of the host. In a recent experiment, feeding grey mullet fingerlings with the prebiotic Immunogen at the level of 2 g kg\(^{-1}\) diet showed no mortality during the trial. Hence, the study conditions were suitable for the cultivation of the grey mullet fingerlings. According to some researches, distinctive dietary prebiotics have advantageous impacts on the growth yield, feed utilization, survival rate and immune response of aquatic animals (Zhang et al. 2014; Soleimani et al. 2015). Our results showed that Immunogen at all treatments (0.5, 1 and 2 g kg\(^{-1}\)) enhance growth parameters. Our results are in line with the other research: Torrecillas et al. (2007) reported a significant gain in body weight and total length as well as a positive correlation between the MOS levels and feed intake in European sea bass \((Dicentrarchus labrax)\) feeding on MOS at different concentrations of 2% and 4%. At the same time, Ebrahimi et al. (2012) pointed out that diets supplemented with 0.5, 1, 1.5 and 2.5 g kg\(^{-1}\) Immunogen led to weight gain, SG and FCR in common carp \((Cyprinus carpio)\) fingerling, Zhang et al. (2014) showed that dietary supplementation of \(Bacillus subtilis\) and fructooligosaccharide enhance the growth yield of juvenile \(ovate pompano, Trachinotus ovatus\). The most common outcomes on feed utilization were acquired in the fish fed 2 g kg\(^{-1}\) Immunogen diet. PER and FCR for all treatments (0.5, 1 and 2 g kg\(^{-1}\)) were found to be 2.68–3.24 and 0.65–0.54, respectively. Our results are in confirmation with those reported in different experiments on the

| Parameters                  | Control | Treatment1 | Treatment2 | Treatment3 |
|-----------------------------|---------|------------|------------|------------|
| Haemoglobin (g/dL)          | 14.26 ± 2.70 \(^a\) | 14.76 ± 1.87 \(^a\) | 16.23 ± 1.40 \(^a\) | 13.56 ± 2.63 \(^a\) |
| Haematocrit (%)             | 54.50 ± 8.47 \(^a\) | 54.76 ± 6.37 \(^a\) | 51.30 ± 5.30 \(^a\) | 51.53 ± 7.70 \(^a\) |
| WBC (x10\(^3\)/mm\(^3\))    | 8.46 ± 1.61 \(^a\) | 10.14 ± 4.68 \(^a\) | 11.9 ± 0.15 \(^a\) | 12.45 ± 7.21 \(^a\) |
| RBC (x10\(^6\)/mm\(^3\))    | 72.45 ± 1.98 \(^a\) | 79.12 ± 1.40 \(^a\) | 86.59 ± 4.80 \(^a\) | 67.59 ± 4.50 \(^a\) |
| MCHC (g/dL\(^{-1}\))        | 43.82 ± 1.74 \(^a\) | 44.13 ± 1.57 \(^a\) | 42.25 ± 0.54 \(^a\) | 42.53 ± 1.33 \(^a\) |
| MCH (pg)                    | 150.14 ± 4.74 \(^a\) | 145.80 ± 12.20 \(^a\) | 148.00 ± 14.11 \(^a\) | 153.42 ± 14.11 \(^a\) |
| MCV (fL)                    | 324.54 ± 15.15 \(^a\) | 302.44 ± 32.96 \(^a\) | 321.04 ± 47.1 \(^a\) | 331.15 ± 34.17 \(^a\) |
| Neutrophil (%)              | 16.33 ± 2.21 \(^a\) | 14.00 ± 1.60 \(^a\) | 12.56 ± 1.51 \(^a\) | 14.00 ± 1.60 \(^a\) |
| Lymphocyte (%)              | 69.53 ± 3.13 \(^a\) | 72.56 ± 3.16 \(^a\) | 73.56 ± 4.72 \(^a\) | 73.00 ± 2.60 \(^a\) |
| Eosinophil (%)              | 3 ± 1 \(^a\) | 2 ± 1 \(^a\) | 2.33 ± 1.5 \(^a\) | 2 ± 0 \(^a\) |

Notes: Within rows values with different superscripts are significantly different \((p < .05)\). The control group was fed with basal diet and the remaining treatments (1, 2 and 3) were fed with 0.5, 1 and 2 g kg\(^{-1}\) concentrations of prebiotic in diets, respectively. Values are mean ± SE.
other species (Zhang et al. 2014). The improved FCR of grey mullet fingerlings which in our research is in agreement with previous findings in such species as hybrid striped bass Morone chrysops × M. saxatilis (Li and Gatlin 2004), rainbow trout Oncorhynchus mykiss (Stajkov et al. 2007).

As for the three levels of Immunogen, no significant effect on the carcass quality of grey mullet was found. Body moisture, ash, protein and fat contents of the grey mullet fingerlings were not significantly differed (p > .05) among all the experimental treatments. This is in line with our results on other fish species, including Atlantic salmon, rainbow trout and common carp (Grisdale-Helland et al. 2008; Dimitroglou et al. 2010).

According to the results, dietary Immunogen did not affect blood parameters when diets contained 0.5–2 g kg⁻¹. However, WBC levels were significantly increased in fish fed dietary Immunogen. similar results have been reported in oligofructose-fed beluga juveniles (Hoseinifar 2011). The increased WBC count results from stress imposed by fish as a result of daily feeding on b-glucan. Harikrishnan et al. (2003) at the same time reported increased WBC counts in C. carpio after herbal treatment with Azadirachta indica. Most outstanding effects included a rise in the glucose levels; however, a change was observed in the glucose level of treatment 3. The enhanced health condition in the grey mullet fingerlings is probably due to the b-glucan and MOS components of the Immunogen. A number of similar outcomes were recorded in previous researches (Santarem et al. 1997; Chang et al. 2003; Ebrahimi et al. 2012).

For commercial aquaculture, stimulation of the immune response of fish through dietary supplements is of great importance (Staykov et al. 2007). In this regard, the innate immune system can play vital role as aquatic animals are frequently vulnerable to numerous opportunistic pathogens and this part of immune response allows the first line of defence for the host (Magnadóttir 2006). It is obvious that those immunostimulants treated fish had increased immune response parameters (Sakai 1999). Lysozyme is characterized with bactericidal activity and as an opsonic activates the complement system and phagocytes to prevent infection and disease (Alexander & Ingram 1992). Here, fish fed with diets supplemented by high Immunogen level (2 g kg⁻¹) showed higher serum total immunoglobulin and serum lysozyme activities compared to those fed by basal diet and diets supplemented with low Immunogen levels (0.5 and 1 g kg⁻¹), respectively. Similarly our results have been attributed to Caspian roach (Rutilus rutilus) fry by Soleimani et al. (2015). The immunostimulatory nature of prebiotics may be attributed to stimulation of the growth of useful bacteria, including lactic acid bacteria and Bacillus spp. (Sang et al. 2011; Zhang et al. 2011), which possess cell wall components, including lipopolysaccharides in turn fraught with immunostimulatory properties (Masahiro 1999; Raa 2002; Chang et al. 2003; Bricknell & Dalmo 2005; Van Hai and Fotedar 2009; Xian et al. 2009).

4. Conclusion

In conclusion, diet supplemented with the suitable level of Immunogen significantly enhanced the nutrition efficiency, growth yield and innate immune of grey mullet fingerlings. In light of the results of this research, to supplement Immunogen at levels of 2 g kg⁻¹, diet is appropriate to feed grey mullet fingerlings.

Disclosure statement

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