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Protective effects of the prebiotic on the immunological indicators of rainbow trout (*Oncorhynchus mykiss*) infected with *Aeromonas hydrophila*

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ABSTRACT

The aim of this study was to investigate the protective effects of dietary administration of commercial prebiotic, Immunogen, on immunological indicators, enzymatic responses and stress tolerance in juvenile (81.65 ± 1.49) rainbow trout (*Oncorhynchus mykiss*) following *Aeromonas hydrophila* infection. The first group of fish was fed with the diet containing 2 g kg^{-1} Immunogen whilst the control group received the diet free of Immunogen. There were three replicates per group. After 6 weeks feeding, the control group were divided into two treatments injected with saline buffer (control), and 1.5×10^8 CFU *A. hydrophila* respectively. The fish fed with the Immunogen supplemented diet were also injected with 1.5×10^8 CFU *A. hydrophila*. Our results revealed that dietary Immunogen increased the level of white blood cell (WBC) and percentage of lymphocyte ($P < 0.05$), however, the level of red blood cell (RBC), Hematocrit (Hct), hemoglobin (Hb) and percent of monocyte decreased in Untreated-Challenged group but unaffected in the group fed with Immunogen ($P < 0.05$). The level of lysozyme, alternative complement, antiprotease activity, total protein, albumin and globulin decreased in Untreated- Challenged group compared to control group. However, there was an increase in the level of lysozyme, alternative complement, antiprotease activity, bactericidal activity, in the Treated- Challenged group compared to other groups ($P < 0.05$). Serum alkali phosphatase (ALT) and aspartate aminotransferase, significantly increased fallowing challenge with *A. hydrophila* but in the Treated-Challenged group, there was no significant difference compared to the control group ($P < 0.05$). Lactate dehydrogenase (LDH) level was not different between groups ($P > 0.05$). Serum cortisol and glucose levels were higher in the challenge group, but these levels were lower in fish under challenge that were fed Immunogen-supplemented diet in contrast to the group fed control diet. The stress responses affected by *A. hydrophila* challenge ($P < 0.05$). Serum sodium, potassium and calcium concentration decreased by *A. hydrophila* exposure ($P < 0.05$), and Immunogen showed protection effect against this change.

1. Introduction

Aeromonas bacteria are found in the water as well as in normal intestinal micro flora of healthy fish [1]. *Aeromonas hydrophila* is a significant pathogen particularly of freshwater aquaculture [2] that commonly cause hemorrhagic septicemia, fin and tail rot and infectious abdominal dropsy as main symptoms [3]. In the past decades, the common method to cure bacterial disease was drug and antibiotic therapy, but incident of antibiotic resistant strain may prevent the usage of antibiotic therapy [4]. Therefore, in this regard there is an increased interest in using feed additives such as environmental friendly immune-stimulants, probiotic and prebiotic, which can improve the immune and health of fish and can increase resistant to viral and bacterial pathogens [5–7].

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Prebiotics are non-digestible and fermentable components that can increase immune responses through different mechanisms [8]. Immunogen is a commercial prebiotic, mainly includes β-Glucan and Mannaoligosaccharides [9], derived from *Saccharomyces cerevisiae* yeast cell wall that was reported to potential impacts in increasing the level of immunity and resistance to infectious pathogens. For example, improved growth performance and immune response was observed in common carp after dietary administration of Immunogen [5]. In another study on rainbow trout, it was reported that the administration of Immunogen improved immune releated gene and humoral innate immune parameters, as well as decreased heat shock protein (HSP70) gene as stress indicator [10].

It has been confirmed that prebiotic especially mannan-oligosaccharide [11] and β-Glucan play a vital role in the cellular and humoral immune mechanisms of host, such as increase serum lysozyme activity, phagocytosis, serum complement activity, that will in turn result in promote disease resistance [12–15]. Most of previous studies have however investigated the effect of prebiotic on hemato-immunological parameters, some serum enzyme and histology in healthy fish [16–18]. But there is limited information about protective effect of prebiotic on health statues of infected fish. In this regard, Torrecillas, Makol [19] have reported that dietary MOS supplementation of diet reduced serum cortisol as most common indicator of stress in *Dicentrarchus labrax* [20] after *Vibrio anguillarum* challenge. There is limited information regarding the effects of Immunogen on the species such as Rainbow trout and the importance of this product in aquaculture. Thus, the aim of the present study was to investigate the protective effects of a single dose of dietary Immunogen on some hematological parameters, serum enzyme activity, stress and innate immune responses of rainbow trout after challenge with *A. hydrophila*.

2. Material and methods

2.1. Fish culture and experimental design

This experiment was carried out on 180 juvenile rainbow trout (81.65 ± 1.49 g) that were obtained from Mahisara fish culture (Karaj, Iran). The fish were transferred to fish disease laboratory at the University of Tehran and they were equally stocked in nine fiberglass tanks (1000 L) assigned to three treatments (I: untreated-unchallenged (control), II: untreated-challenged, III: treated-challenged with Immunogen (commercial prebiotic, ICC Co; USA), in three replicates. They acclimatized to laboratory condition for 14 days and during acclimation they were fed (2% of body weight) with a commercial rainbow trout diet (Behparvar Co; Iran, Tehran) twice a day before the onset of the experiment. At first, the fish were fed with the experimental diets for 6 weeks, and then the treatment groups were injected with *A. hydrophila* (II and III) or saline buffer (I). On the 6th week of feeding, fish reared in groups II and III were injected based on the LD_{50} of *A. hydrophyla* for rainbow trout (1.77×10^7 to 4.9×10^7 CFU) which previously determined by LaPatra et al. [21] and again was tested by Ahmadi, Farahmand [22] for *A. hydrophila* (RTCC1032). Group I (control) was injected with saline buffer as negative control and were kept three weeks post challenge. During the experimental trial (9 weeks) fish were fed (2% of body weight) twice daily (09:00 and 17:00). Water temperature, dissolved oxygen and pH were monitored daily and maintained at 18 ± 1.2 °C, 6.6 ± 0.3 mg L^{-1} and 6.9 ± 0.4, respectively.

2.2. Diet preparation

Based on producer company suggestion (ICC Co; USA), two isonitrogenous and isocaloric diets were formulated (Table 1) containing 0 g kg^{-1} (control group) and 2 g kg^{-1} Immunogen, and tested in triplicate. All the dietary ingredients were blended thoroughly in a mixer and then the ingredients were blended with water to form a paste which was then passed through a meat grinder equipped with a 4.8 mm die to obtain uniform pellets. The pelleted diets were dried at 40 °C and stored in plastic bags at −4 °C until use.

Table 1
Ingredients and proximate composition (g kg^{-1} dry matter) of formulated diets.

Ingredients	Supplemented diet	Control diet
Fish meal[a]	455	455
Soybean meal	179	180
Corn starch	211.1	213
Soybean oil	48.8	48.8
Fish oil	23.1	23.1
Vitamin[b]	30	30
Mineral[c]	30	30
CMC[d]	20	20
Immunogen	**2**	**0**
Proximate composition		
Crude protein	450.8	453.2
Crude lipid	91.4	91.4
Ash	110.2	110.2
Fibre	2.2	2.2
NFE[e]	347.4	347.4
GE[f](Mj/kg)	20.41	20.71

[a] Danish fish meal.
[b] Vitamin contains (kg^{-1} dry weight), Vitamin A: 50.000 MIU, Vitamin D3: 10 MIU, Vitamin E: 130 g, Vitamin K3: 10 g, Vitamin B1: 10 g Vitamin B2: 25 g, Vitamin B6: 16 g, Vitamin B12: 100 mg, Niacin: 200 g, Pantothenic acid: 56 g, Folic acid: 8 g, Biotin: 500 mg, Antioxidant: 0.2 g, Anti-cake: 20 g.
[c] Mineral premix, contains (kg^{-1} dry weight): calcium phosphate 397 g; calcium lactate 327 g; ferrous sulphate 25 g; magnesium sulphate 137 g; potassium chloride 50 g; sodium chloride 60 g; potassium iodide 150 mg; copper sulphate 780 mg; manganese oxide 800 mg; cobalt carbonate 100 mg; zinc oxide 1.5 g; sodium selenite 20 mg.
[d] Carboxyl Methyl Cellulose (sodium salt), binder.
[e] Nitrogen free extract calculated as 1000-(protein + lipid + ash + fiber) g kg^{-1}.
[f] Gross energy calculated based on 17.2, 39.5 and 23.6 Kj/g for carbohydrate, lipid and protein respectively.

2.3. Bacteria preparation and challenge test

A. hydrophila (RTCC1032) provided by the Department of Aquatic Animal Health, Faculty of Veterinary Medicine, University of Tehran, Tehran, Iran and was tested for pathogenesis. Stock of *A. hydrophila* were cultured in tryptic soy broth media (TSB: w/v; Merk, Germany) and stored at −70 °C in 0.85% NaCl with 20% glycerol (v/v) to provide stable inoculate throughout the experiment [23]. *A. hydrophila* subculture was taken on TSA slope and harvested in physiological saline (0.85%). Optical density of 1.0 at a wavelength of 640 nm corresponds to a bacterial concentration of 1.55×10^9 colony forming units (CFU) mL^{-1} [21]. Previously, LD_{50} of *A. hydrophyla* for rainbow trout (1.77×10^7 to 4.90×10^7 CFU) has been determined by LaPatra et al. [21] and tested by Ahmadi, Farahmand [22] for *A. hydrophila* (RTCC1032) by inoculation into rainbow trout.

2.4. Blood sampling

At the end of feeding trial (i.e. 8 weeks), all fish groups were starved for 24 h before sampling and then fish were anesthetized by using clove powder (200 ppm). The blood was collected by venipuncture from caudal vein. A half of blood samples were transferred to heparinized tubes to assay hematological parameters. Other sections of blood samples were transferred to non-heparinized tubes to clotting and separation of serum. Then,