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# The effects of dietary Immunogen® on innate immune response, immune related genes expression and disease resistance of rainbow trout (*Oncorhynchus mykiss*)

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ABSTRACT

This study investigates the effects of prebiotic Immunogen® on lysozyme, TNFα and HSP70 gene expression in head kidney, humoral innate immune parameters and resistant to *Aeromonas hydrophila* of rainbow trout. 120 healthy rainbow trout (81.65 ± 1.49 g) were distributed in six fiberglass tanks assigned to two groups fed control or diet supplemented with 2 g $kg^{-1}$ Immunogen® for 45 days. The results revealed that administration of Immunogen® significantly ($P < 0.05$) up regulated lysozyme and TNFα gene expression. HSP70 gene expression was significantly ($P < 0.05$) lower in Immunogen® fed fish at the end of trial. Humoral innate immune parameters (lysozyme activity, ACH50 and bactericidal activity) were significantly ($P < 0.05$) increased whether 15 or 45 days after feeding on Immunogen® supplemented diet. However, significant ($P < 0.05$) increase in agglutination antibody titer observed just after 45 days feeding on Immunogen®. Rainbow trout fed with 2 g $kg^{-1}$ Immunogen® showed remarkably higher resistance against *A. hydrophila* (64.44% survival) compared to the control group (24.44% survival). These results confirm that Immunogen® can up regulates immune related genes expression, stimulates immune response that per se enhances disease resistance in rainbow trout.


## 1. Introduction

The release of disinfectants and drugs into the rearing water of the intensive fish production system, can affect the integrity of gastrointestinal microbiota [1], decreases growth performance and suppresses the immune responses [2,3]. Also, the administration of antibiotics may results in food and environmental pollutions and development of antibiotic resistant pathogens [4–7]. Along with tremendous growth of world aquaculture during the last years, there was increasing interests toward administration of ecological and environmental friendly approaches like immunestimulants, probiotic and prebiotic as a replacement for antibiotic therapy [8–11].

Prebiotics are "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" [12]. The positive effects of prebiotics on immune response and resistance of fish and shellfish is well-documented [8,9,11,13] and previous studies demonstrated that prebiotics can stimulate both innate immune mechanisms and cells involved in the specific immune response [13–16].

Immunogen® is a commercial prebiotic, mainly includes β-Glucan and mannaoligosaccharides (MOS), derived from *Saccharomyces cerevisiae* yeast cell wall [17]. MOS and β-glucan have been demonstrated to show beneficial effects on specific and non-specific immune mechanism of monogastric animal [8,17–19]. Ebrahimi et al. [20], reported improved growth performance and immune response of common carp after administration of Immunogen® in diet. Despite recent progress in the field of prebiotic administration in aquaculture [8] there is limited information about the effects of administration of Immunogen® [20] as well as genetic insight into the effects of prebiotics on fish immune system. Thus, the aim of the present study was determination of dietary Immunogen® effects on some hematological parameters, humoral immune response, immune related genes expression (Lysozyme (lyz2), tumor necrosis factor α (TNFα), heat shock protein 70

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(HSP70) and resistance of rainbow trout against *Aeromonas hydrophila*.

## 2. Material and methods

### 2.1. Fish culture and feeding trial

Hundred and twenty rainbow trout (81.65 ± 1.49 g) obtained from Mahisara fish culture (Karaj, Iran) and transferred to fish disease laboratory of University of Tehran. After checking the health status, they were equally stocked in six fiberglass tanks (1000 L) assigned to two levels (0 and 2 g $kg^{-1}$) of Immunogen® (commercial prebiotic, ICC Co; USA) with three replicates. The fish were acclimatized to laboratory condition for 10 days and during acclimation were fed (2% of BW) with a commercial rainbow trout diet (Behparvar Co.) twice a day. During the experimental trial (5 weeks) fish were hand-fed (2% of BW) twice daily (09:00 and 17:00). The feeding ration was corrected every 2 weeks following a 24-h starvation period and batch weighing. 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

A single dose of Immunogen® (2 g $kg^{-1}$) was incorporated in a basal diet formulated for rainbow trout (crude protein: 45.3%; crude lipid: 9.1%; Gross energy: 20.4 Mj $kg^{-1}$). The dietary 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 further use.

### 2.3. Hematological parameters

Fish were anesthetized for hematological analysis by using clove powder (200 ppm, 20 min), and blood samples were taken from caudal vein. Blood samples were transferred to heparinized tubes and Red blood cells (RBCs) and white blood cells (WBCs) were counted on haemocytometer slide according to Sarder et al. [21]. Hematocrit was determined by the microhaematocrit method as described by Brown [22] and reported as percentage packed cell volume (% PCV). Hemoglobin levels were estimated using Sahli's method [23].

### 2.4. Immune relates genes expression

#### 2.4.1. Total RNA extraction and cDNA synthesis

RNA extraction of head kidneys were carried out by the acid guanidinium thiocyanate-phenolchloroform method described by Chomczynski and Sacchi [24]. Total RNA extraction was performed according Awad et al. [25] with slight modifications. Head kidney tissue samples were obtained and immediately transferred to liquid nitrogen tank and then kept in −80 °C until further use. For total RNA extraction 50–100 mg of tissue homogenated in 1.0-mL Biozole reagent (Bio flux; China) for 15 min in room temperature following the manufacturer protocol. The quantity and concentration of RNA were measured by spectrophotometer at 260/280 nm. Also, the RNA quality was evaluated by electrophoresis on a 1.5% agarose gel and staining with ethidium bromide [26]. 1 μg of total RNA was used to synthesize first-strand cDNAs using a Fermentas cDNA synthesis Kit for RT-PCR, following the manufacturer's instructions and a mixture of oligo-dT as primer.

#### 2.4.2. Primer design

The qPCR primers for *HSP70*, *Lyzo2* and *TNFα* genes were designed based on the conserved regions extracted from DNA sequences in GenBank and the genomic contigs of the NCBI database. Multiple qPCR primer combinations were designed for each gene using PRIMER3.0 software program (available at http://www-genome.wi.mit.edu) (Table 1). The qPCR efficiency was also taken into account for selection of the best qPCR primer pair with specific and correct size. β actine gene was used as reference gene for standardization of expression levels.

#### 2.4.3. Quantitative real-time PCR (qPCR)

Real-time PCR analysis was carried out using an iCycler (Bio-Rad) with Fermentas Maxima SYBR Green qPCR Master Mix (2×) (Fermentas) and all primers at [100 nM] after protocol suggested by Miandare et al. [26]. The fold change in *HSP70*, *Lyzo2* and *TNF* relative mRNA expression was calculated by the $2^{-\Delta\Delta Ct}$ method [27]. The obtained data were analyzed using the iQ5 optical system software version 2.0 (Bio-Rad).

### 2.5. Immunological assays

Alternative complement activity was determined based on the hemolysis of sheep red blood cells (SRBC) according to Ortuno et al. [28] protocole. The volume of serum yielding 50% hemolysis was determined and used to calculate the complement activity of the samples (value of ACH50 is in units per ml). Serum lysozyme activity was measured after Demers and Bayne [29] based on turbid metric assay and lysis of the lysozyme-sensitive Gram-positive bacterium *Micrococcus luteus* (Sigma).

The serum bactericidal activity was determined according to the method which previously described [30] with some modification. *A. hydrophila* (RTCC1032) isolates were adjusted to 1.0 ($OD_{540}$; $10^7$ CFU $ml^{-1}$). Bacterial suspension and serum samples were mixed with 1:1 ratio and incubated for 90 min at 25 °C. Thereafter, 10 μL of serum and bacteria mixtures were transferred to the Tryptic soy agar (TSA) medium and cultured for 24 h at 25 °C. Phosphate buffer saline (PBS, Sigma) was used instead of serum as negative control. Viable colonies were counted and results expressed as percentage of survival in the PBS controls.

The agglutination antibody titer was conducted in round bottomed 'U' shaped micro titer plates after Swain et al. [31] with slight modifications. 50 μl of serum was serially diluted in PBS (1/2, 1/4, 1/8, 1/16 and 1/32) and then equal volumes of *A. hydrophila* (RTCC1032) (1.0 $OD_{540}$; $10^7$ cells $ml^{-1}$) was added to wells and kept for 24 h at room temperature. The reciprocal of the highest dilution that gave agglutination was taken as the agglutination antibody titer which is expressed as $\log_2$.

### 2.6. Disease resistance

The experimental disease resistance test was carried out based on the $LD_{50}$ of *A. hydrophyla* for rainbow trout ($1.77 \times 10^7$ to $4.90 \times 10^7$ CFU) which previously determined by LaPatra et al. [32]. At the end of feeding trial, ten fish per each replicate transferred to new tank and intraperitoneally (i.p.) injected with 0.1 ml PBS suspensions containing *A. hydrophila* (RTCC1032). All groups were kept under observation for 10 days to record clinical signs and mortality rate. The relative percent of survival (RPS) was calculated 10 days post challenge according to following formula:

$$RPS = 100 - [(\text{test mortality}/\text{control mortality})*100]$$

**Table 1**
Sequences of oligonucleotide primers and the conditions used for real-time PCR.

| Gene | Accession number | qPCR primers, forward/reverse | Amplicon (bp) |
|---|---|---|---|
| Lysozyme C-type | NM_001124374.1 | ACAGCCGCTACTGGTGTGACG<br>GCTGCTGCCGCACATAGAC | 203 |
| TNF1α | NM_001124374.1 | CAAGAGTTTGAACCTTGTTCAA<br>GCTGCTGCCGCACATAGAC | 180 |
| HSP70 | AB062281.1 | CTGCTGCTGCTGGATGTG<br>GCTGGTTGTCGGAGTAAGTG | 200 |
| β actine | NM_001124235.1 | ATGGGCCAGAAAGACAGCTACGTG<br>CTTCTCCATGTCGTCCCAGTTGGT | 140 |

**Table 2**
Hematological parameters of rainbow trout fed control diet or diet supplemented with 2 g kg$^{-1}$ Immunogen® for 5 weeks. Values in a row with different superscripts denote significant difference ($P < 0.05$). Values are presented as the mean ± SD.

| Hematological parameter | 15th day of experiment | | 45th day of experiment | |
|---|---|---|---|---|
| | Control | 2 g kg$^{-1}$ Immunogen® | Control | 2 g kg$^{-1}$ Immunogen® |
| RBC | 1.44 ± 0.14 | 1.57 ± 0.12 | 1.45 ± 0.13 | 1.69 ± 0.20 |
| WBC | 3.31 ± 0.32 | 3.63 ± 0.41 | 3.21 ± 0.34$^{a}$ | 4.28 ± 0.31$^{b}$ |
| Hb | 10.33 ± 0.47 | 10.73 ± 0.20 | 10.23 ± 0.75 | 11.16 ± 0.64 |
| Hct | 38.00 ± 3.00 | 42.33 ± 2.08 | 39.50 ± 1.50$^{a}$ | 44.16 ± 1.25$^{b}$ |

### 2.7. Statistical analysis

Data were analyzed using independent sample *T* test. Mean values were considered significantly different at $P < 0.05$. Prior to statistical analysis, normality and homogeneity of variance were checked and percentage data were subjected to arcsine transformation. All statistical analyses were conducted using SPSS statistical package version 16.0 (SPSS Inc., Chicago, IL, USA). Data are expressed as mean values ± SD.

## 3. Results

The hematological parameters of rainbow trout fed diet supplemented with 0 or 2 g kg$^{-1}$ prebiotic Immunogen® are presented in Table 2. No significant differences ($P > 0.05$) observed in hematological parameters after 15 days feeding on experimental diets. However, at the end of trial WBC and Hct were significantly higher ($P < 0.05$) in 2 g kg$^{-1}$ Immunogen® fed fish compared to the control group.

The effects of Immunogen® on the expression of immune related genes; lysozyme and TNFα of rainbow trout are shown in Fig. 1. The present study revealed that feeding on 2 g kg$^{-1}$ dietary Immunogen® (either after 15 or 45 days) significantly ($P < 0.05$) increased the expression of immune related genes; lysozyme and TNFα. The relative expression of HSP70 gene (Fig. 2) showed no significant ($P > 0.05$) differences between control and Immunogen® after 15 days feeding on experimental diets. However, significantly lower ($P < 0.05$) HSP70 expression observed in fish received 2 g kg$^{-1}$ prebiotic immunogen® at the end of feeding trial.

The effect of dietary prebiotic Immunogen® on the humoral innate immune parameters; alternative complement activity, lysozyme activity, serum bactericidal activity and agglutination antibody titer are presented in Fig. 3. Dietary Immunogen® significantly ($P < 0.05$) elevated serum complement and lysozyme activity of rainbow trout. Likewise, significant ($P < 0.05$) difference observed between serum bactericidal activity of control group and Immunogen® fed fish (whether after 15 or 45 days feeding). Whereas, agglutination antibody titer showed significant ($P < 0.05$) differences only after 45 days feeding on Immunogen® supplemented diet.

The cumulative mortality of rainbow trout fed Immunogen® supplemented or control diet during 10 days post challenge with *A. hydrophila* is shown in Fig. 4. Monitoring the fish mortality for post challenge revealed that mortality was remarkably lower in the Immunogen® fed group (36.66 ± 5.77%) compared to the control group (76.66 ± 15.27). The relative percent of survival (RPS) was 52.17% in fish fed 2 g kg$^{-1}$ Immunogen®.

## 4. Discussion

It is well documented that administrations of dietary prebiotics stimulate immune response, elevate disease resistance and improve growth performance of fish and shellfish [8,11]. Stimulation of the immune response of fish through dietary supplements is of high interest for commercial aquaculture [17]. To our knowledge, this study is the first attempt to investigate the effects of Immunogen® as a prebiotic immune gene expression, humoral immune response and disease resistance of rainbow trout. The results of the present study showed that WBC and Htc were significantly higher in Immunogen® fed fish. Similar to these results inclusion of 1.5 and 2.5 g kg$^{-1}$ prebiotic Immunogen® in common carp diet resulted in significant increase of WBC and administration

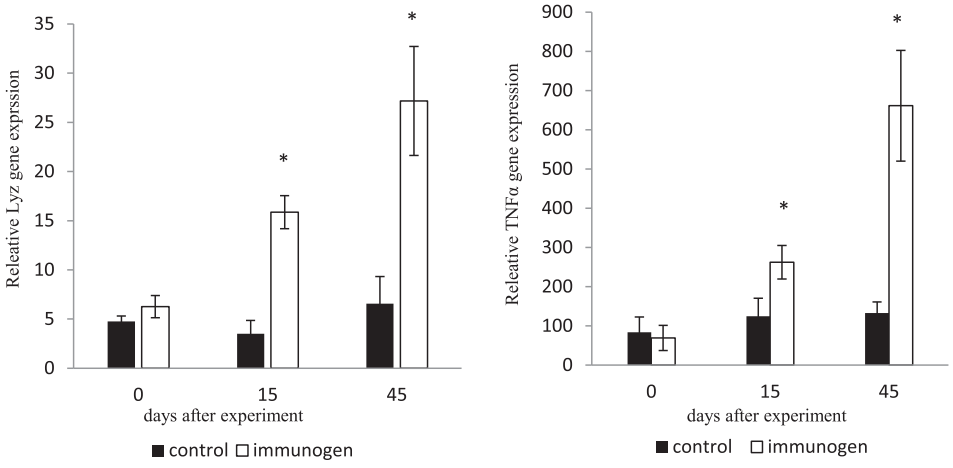


**Fig. 1.** The relative expression of immune related genes; lysozyme and TNFα in head kidney of rainbow trout fed 2 g kg$^{-1}$ prebiotic Immunogen®. Data are expressed as the mean ± SD. The bar in each sampling marked with asterisks shows significant different ($P < 0.05$).

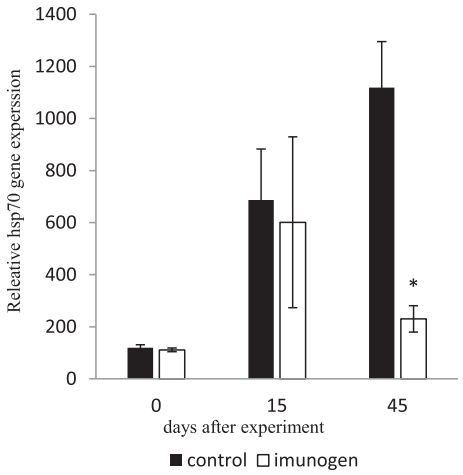


**Fig. 2.** The relative expression of HSP70 gene in head kidney of rainbow trout fed 2 g $kg^{-1}$ prebiotic Immunogen®. Data are expressed as the mean ± SD. The bar in each sampling marked with asterisks shows significant different ($P < 0.05$).

of 0.5 g $kg^{-1}$ elevated Htc compared to other groups [20]. The higher WBC levels observed in the present study hint at a possible immunomodulatory effect which per se confirmed by up-regulation of immune related gene expression.

Innate immune system in fishes plays a key role in preservation of fish against infectious diseases [10]. Lysozyme (muramidase, EC 3.2.1.17) is a lytic enzyme that hydrolyzes glycosidic bonds and its level or activity has been commonly measured as an important innate immune parameter [33]. Administrations of 2 g $kg^{-1}$ Immunogen® in the present study up-regulated lysozyme gene expression and increased serum lysozyme activity of rainbow trout. There was no published data on the effects of immunogen® on immune statues of rainbow trout. Therefore we compared the results with those obtained from administration of mannanoligosaccharides (MOS) and β-glucan which are the main constitute of Immunogen®. In line with our results, Staykov et al. [18] reported that administration of 2 g $kg^{-1}$ MOS elevated serum lysozyme activity in rainbow trout. Similarly, bacterial lipopolysaccharide (LPS) and beta-glucan injection increased lysozyme gene expression in head kidney and serum lysozyme activity of Atlantic salmon (*Salmo salar* L.) [34].

The tumor necrosis factor gene in rainbow trout gill and kidney has been sequenced previously as 2007 bp length include four exons region and three introns [35]. Sealey et al. [36] stated that inclusion of Grobiotic-A, a commercial prebiotic contains a mixture of partially autolyzed brewer's yeast, dairy components and dried fermentation products, up-regulated TNFα gene expression in rainbow trout, that was in accordance to the results of this study. However, in contrast with these results, β-glucan (derived from *S. cerevisiae*) had no significant effects on the expression of TNFα in zebra fish (*Danio rerio*) [37]. These differences can be attributed to the prebiotic source, dosage and the species. Also, in a probiotic study, Panigrahi et al. [38] observed up regulation of TNFα gene

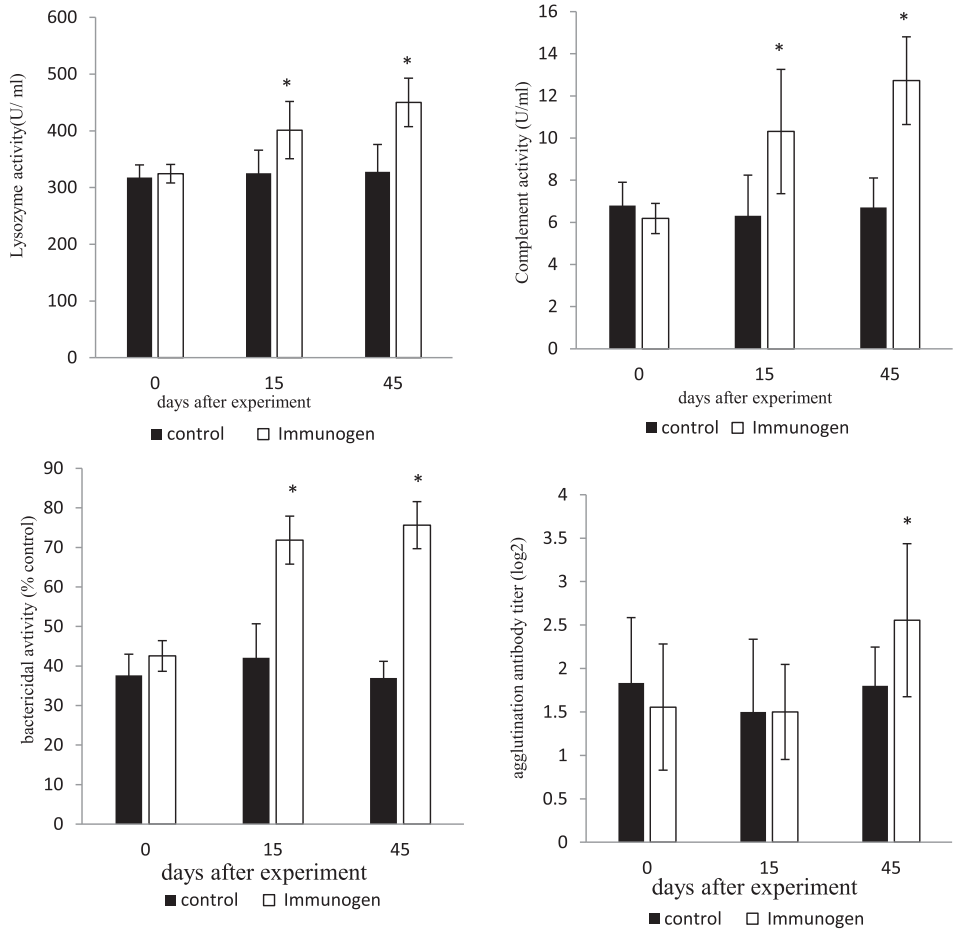


**Fig. 3.** The serum lysozyme, complement, bactericidal activity and agglutinating antibody titer of rainbow trout fed 2 g $kg^{-1}$ prebiotic Immunogen®. Data are expressed as the mean ± SD. The bar in each sampling marked with asterisks shows significant different ($P < 0.05$).

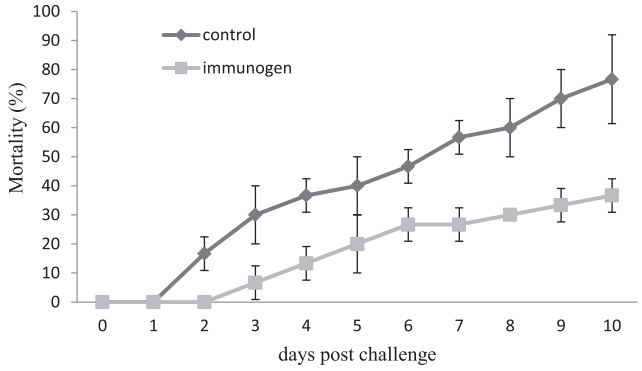


**Fig. 4.** The cumulative mortality of rainbow trout fed 2 g $kg^{-1}$ prebiotic Immunogen® during 10 days post challenge with *A. hydrophila*. Values are presented as the mean ± SD.

expression in rainbow trout after feeding with *Lactobacillus rhamnosus*. It seems that probiotic and prebiotic affect the innate immune system through the same mechanism of gut microbiota modulation [39], Although, the mechanism of action of dietary Immunogen® on immune gene expression is not clear but β-glucan (one of the main constitutes of Immunogen®) appears to be able to stimulate the production of proinflammatory cytokines and chemokines [37].

The HSP70 gene expression in the head kidney of rainbow trout remarkably down-rehulated following administration Immunogen® in diet, as shown in Fig. 2. The heat shock protein 70 has several functions and expressed in response to a wide range of stressors [40]. Although there was no published information about the effects of prebiotic on HSP70 gene expression but Rollo et al. [41] demonstrated that *Lactobacillus fructivorans*, isolated from sea bream (*Sparus aurata*) gut, and *Lactobacillus plantarum*, isolated from human faeces down-regulated HSP70 expression in sea bream. Likewise, administration of probiotic *Lactobacillus rhamnosus* IMC 501 caused down-regulation HSP70 expression in clownfish (*Amphiprion ocellaris*) larvae [42]. Down-regulation of HSP70 expression is possibly due to elevated tolerance toward usual stresses during culture like stresses caused by sampling for monitoring water quality, fish biometry or other unwanted stresses. Further studies are necessary to elucidate the mechanism by which dietary Immunogen® affect stress tolerance and HSP70 gene expression in rainbow trout.

The humoral immune parameters were measured in the present study as an indicator of health status and innate immune parameters of rainbow trout fed Immunogen®. The complement activity is one of the most important components of innate immune system in fish and plays a key role in the lysis and opsonization of foreign cells and organisms [43]. Agglutination antibody titer against different bacteria is another mechanism of innate immune response which has high activity in fish [44]. As shown in Fig. 3, fish fed 2 g $kg^{-1}$ Immunogen® had significantly greater alternative complement (ACH50), serum lysozyme, bactericidal activity and agglutination antibody titer. In agreement with the results of the present study, Ebrahimi et al. [20] reported that Immunogen® improved innate immune parameters of common carp. Also previous studies confirmed positive effects of dietary MOS on innate immune parameter of rainbow trout [18,45], European sea bass, *Dicentrarchus labrax* [46] and juvenile Jian carp, *Cyprinus carpio* Var. Jian [47]. Although the mechanisms of action of Immunogen® on fish immune system is not clear but it has been suggested that MOS stimulates mannose binding lectin (MBL) by liver secretion that binds the capsule of bacteria and triggers the complement cascade [48].

The results of the present study revealed that Immunogen® significantly increased rainbow trout resistance to *A. hydrophila*. Similarly, common carp fed Immunogen® showed greater resistance (survival %) after challenge with *A. hydrophilla* [20] and Lin et al. [49] reported that dietary brewer's yeast and prebiotic Grobiotic™ significantly increased resistance of juvenile rainbow trout against *Streptococcus iniae*. The elevation of disease resistance in the present study is possibly due to up-regulation of immune related genes and stimulation of humoral immune response which are demonstrated above.

In summary, the results of the present study revealed that administration of 2 g $kg^{-1}$ Immunogen® up-regulated lysozyme and TNFα gene and down-regulated HSP70 gene expression. Also, dietary Immunogen® increased humoral immune response that per se resulted in higher resistance in disease challenge test. However, evaluations of the mechanisms of action, effects on the indigenous gut microbiota (autochthonous and allochthonous) and gut morphology studies merits further investigations.

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