Effects of dietary probiotic, *Lactobacillus casei* on some hematological and immunological parameters in Rainbow trout (*Oncorhynchus mykiss*) fingerlings

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Abstract

A 60-day feeding trial was conducted to examine the effects of dietary *Lactobacillus casei* (PTCC 1608) on some hematological and immunological parameters of Rainbow trout (*Oncorhynchus mykiss*). Four supplementation diets at the levels of 0, $5 \times 10^6$, $5 \times 10^7$ and $5 \times 10^8$ CFU g$^{-1}$ of *Lactobacillus casei* were fed to Rainbow trout fingerlings (average weight $32.6 \pm 5.5$ g) in three replicates. Fish were hand-fed three times daily to apparent satiation. The results showed that no significant changes in hematological parameters and immunological indices such as NBT and complement activity. The highest activity rate of serum lysozyme activity was seen in T2 group ($P<0.05$). Concluded that *Lactobacillus casei* fed to the rainbow trout, with the quantity of $5 \times 10^6$ and $5 \times 10^7$ CFU g$^{-1}$, can improve the performance of the immune system, but it is no effect on hematological indices.

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Introduction
Aquaculture practices have been done using high-quality diets with high protein content which should contain not only necessary nutrients but also supplementary additives to keep organisms healthy and result in good growth performance. Aquaculture industry is faced with the challenges of inadequate supply and high costs of quality fish feeds. Commercial fish feeds usually contain fish meal as the major protein source, ranging from 30-50% (Hardy, 1995). Apart from the high costs of fish feeds, disease outbreak is a major challenge in fish farming. In the past, antibiotics had been frequently used to enhance growth and/or resistance to disease in aquaculture systems. The traditional use of antibiotics as growth promoters in aquaculture has been challenged by the potential development of antibiotics-resistant bacteria, the presence of antibiotic residues in food productions, the destruction of microbial populations in the aquacultural environments and the immune suppression of the aquatic animals (Sapkota et al., 2008). Probiotics have been used as growth promoters to replace the widely-used antibiotic and synthetic feed supplements (Fuller, 1989). Probiotic treatment may be particularly useful to secure the settlement of fish intestinal microbiota, and it may improve health in fish (Gatesoupe, 1999). According to Moriarty (1998), probiotics are beneficial bacterial cultures, added to water or fish feeds, which can subsequently improve the health of the host. The range of probiotics examined for use in aquaculture has encompassed both gram-negative and gram-positive bacteria, bacteriophages, yeasts and unicellular algae (Irianto and Austin, 2002; Pooramini et al., 2009). Rainbow trout is one of the most important commercial species in Salmonidae, which are grown widely in many countries. Now, this fish have valuable share in the human food supply (Talebi et al., 2013) but this species faced many pathogens and various stressors in culture systems cause disease and weakened immune system. Up so far, no study has been carried out to investigate the effects of Lactobacillus casei on hematological and immunological parameters of rainbow trout. Therefore, this study was designed to examine the effects of Lactobacillus casei on hematological and immunological parameters of rainbow trout.

Materials and methods
Experimental Design
The present experiment was conducted in 12 cement ponds for 60 days. Healthy rainbow trouts (400 fish in each tank) were obtained from Zagros Mahi breeding center (Izeh, Khuzestan, Iran). Fish of the same size (32.6 ± 5.5 g) were randomly distributed in four experimental groups with each of the three replicates. Prior to the start of the experiment, the fish were fed with the control diet for 7 days for acclimation period. During the experimental period water parameters were measured every day, water temperature 16 ± 1°C, dissolved oxygen 8 ± 0.5 ppm and the pH 7.9 ± 0.2.

Experimental diets
The control group was fed with a non-supplemented basal diet during the entire trial period. Treatment 1 (T1), treatment 2 (T2) and treatment 3 (T3) were fed with diets containing viable probiotic bacteria of different concentrations, $5 \times 10^6$, $5 \times 10^7$ and $5 \times 10^8$ CFU g⁻¹ L. casei respectively. To reach the final concentration, probiotics were slowly applied and mixed into the diets.

Hematological parameters
At the end of the 60 days of nutrition trial, blood samples (15 fish from each treatment) were taken from the caudal vein for hematological and immunological analyses. Blood was sampled by puncturing the caudal vein using a 2 ml syringe. Prior to blood sampling, the fish were anaesthetized with clove powder (150 ppm). For hematological measuring, heparin was used as an anti-coagulant at a concentration of 5,000 IU–heparin sodium salt in 1 ml. Hematological examination was carried out immediately after sampling to assess indices of the leukocyte profile. The number of leukocytes (WBC) was determined in the blood diluted in Natt–Herrick solution using a Neubauer haemocytometer. The WBC number was determined on each blood smear and calculated. The total red blood cell count of the
fish was done manually using the Neubauer Haemocytometer lam and the cell count was multiplied by the dilution factor (10000). The total red blood cell number per mm$^3$ of blood was calculated (Thrall, 2004). The cell indices were calculated using the formula mentioned by Thrall (2004).

$$\text{MCV (μm}^3/\text{cell}) = \frac{\text{Packed cell volume as percentage}}{\text{RBC (millions cell mm}^3)} \times 10$$

$$\text{MCH (pg/cell)} = \frac{\text{Hb (g/100 ml)}}{\text{RBC (millions cell mm}^3)} \times 10$$

$$\text{MCHC (g 100/ml Hct)} = \frac{\text{Hb (g/100ml) /packed cell volume (％)}}{100}.$$

**Immunological parameters**

To measure the Nitro Blue Tetrazolium (NBT), the method recommended by Kajita et al. (1990) was used with some changes. To measure the complement activity, the hemolysis test was used in agar gel (Brata et al., 1993). To evaluate the lysozyme activity, the method of agarose lysoplate and powder of *Micrococcus lysodeikticus* bacteria (Sigma) recommended by Roed et al. (1993) were used.

**Statistical analysis**

The effects of *Lactobacillus casei* on hematological and immunological parameters of the rainbow trout were tested by one-way ANOVA. When significant differences were found, the Duncan’s post hoc test was used to rank the groups. All statistical analyses were performed using the SPSS version 16 with a significant level of $P < 0.05$. The values presented are mean ± standard error (SE).

**Results**

The results of hematological parameters are shown in Table 1. There was no difference in the number of white blood cells (WBC) in different treatments ($P>0.05$). The mean Corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC) were not affected by various concentrations of *Lactobacillus casei* ($P>0.05$).

**Immunological factors** such as NBT, complement and lysozyme activity are presented in Table 2. As can be observed, there is no significant differences in the serum bactericidal activity (NBT) ($P>0.05$). Serum complement also showed no significant differences in the experimental groups ($P>0.05$). Groups T1 and T2 showed an increasing trend in serum lysozyme levels and were significantly higher than control group ($P<0.05$). The highest lysozyme was 130 ± 2.8 units ml$^{-1}$ in T2 and the lowest was in 102.5 ± 4.8 units ml$^{-1}$ in Control.

**Discussion**

*Lactobacilli* sp. are a group of lactic acid bacteria, gram-positive, rod-shaped, non-spore forming, catalase-negative, oxidase negative, usually without moving and not revive nitrate. *Lactobacilli*, with more than 100 species are considered as the biggest lactic acid bacteria genus. Species belonging to this genus are very heterogeneous by having phenotypic, biochemical, physiological and genetic diversity. Members of this genus are found in the natural habitats rich in carbohydrates or proteins such as...
plants and spoiled foods. Moreover, many of them are normal flora of the gastrointestinal and genital tract of human and animals. Because of the long history of their use in brewing and food industries as well as the lack of pathogenicity are generally recognized as the safe organisms (Ringo and Gatsop, 1998; Awal and Palwa, 2005). Lactobacilli for the first time was removed by Dier (1974) from the skin, gills and digestive tract of Atlantic cod (Gadus morhua). Then, gradually, the researchers could separated some species of Lactobacillus from the gut of most teleost at all stages of life, including the larval, fingerling and adult.

**Table 2. Assessing the immunological factors of rainbow trout at the end of the trial period.**

<table>
<thead>
<tr>
<th>Diets</th>
<th>Con</th>
<th>T1</th>
<th>T2</th>
<th>T3</th>
</tr>
</thead>
<tbody>
<tr>
<td>NBT</td>
<td>92.25±13.94</td>
<td>101.00±9.555</td>
<td>109.50±10.085</td>
<td>89.50±9.525</td>
</tr>
<tr>
<td>Complement activity</td>
<td>25.53±7.295</td>
<td>20.20±5.755</td>
<td>24.00±0.61</td>
<td>24.78±4.905</td>
</tr>
<tr>
<td>Lysozyme</td>
<td>102.5±4.8</td>
<td>127.5±3.25</td>
<td>130±2.8</td>
<td>110±4.6</td>
</tr>
</tbody>
</table>

In relation to the hematological indices like WBC, MCV, MCH and MCHC should be stated there are no significantly differences among different treatments. Temperature changes, viral and bacterial diseases and stocking density can cause stress in fish and thereby the WBC. Probably supplemented with probiotic treatments in this study can reduce the stress on the fish because of the probiotic properties.

Serum bactercidial activity of different dose of Lactobacillus were compared and resulted in no significant differences between the control and other treatments. Some reports have reported a lack of serum bactercidial activity after administration of immunstimulants (Cajita et al., 1990; Divyagnaneswari et al., 2007). There was also not found a significant increase in the complement activity in treatments were fed with Lactobacillus casei compared to the control. Although the activity of complement is useful to the immune but continuous activity of the complement system can lead to improper effects and even the suppression of immune system (Taylor et al., 1998).

In the present study, serum lysozyme activity had significantly higher amount in T1 and T2 compared to the control group. Lysozyme activity is an important parameter in defence system of both vertebrates and invertebrates (Sitja-Bobadilla et al., 2008). Giri et al. (2013) reported that lysozyme activity in Rohu (Labeo rohita) was significantly increased in different concentrations of Lactobacillus plantarum which corresponded with the present study.

**Conclusion**

Lactobacillus casei in concentrations of $5 \times 10^6$ and $5 \times 10^7$ CFU g$^{-1}$ (Bacteria number per gram of diet) improved the immune indices of fish, but did not have a significant effect on hematological parameters. Probably can be obtained better results by increasing the concentration of bacteria in the diet or longer periods of oral administration. The addition of this bacterium was ineffective in the rate and level of hematopoiesis, damage and destruction of blood cells. Actually, the use of dietary supplements that are effective to immune enhancing, are the strategies that in addition to provide nutrients needed for growth and development could be useful for improving healthy, resistance to stress and pathogens.

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