

Original Article

Influence of probiotic, *Lactobacillus plantarum* on serum biochemical and immune parameters in vaccinated rainbow trout (*Oncorhynchus mykiss*) against streptococcosis/lactococcosis

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Abstract: This study evaluated the effects of probiotic, *Lactobacillus plantarum* on serum biochemical and some immune parameters of immunized rainbow trout weighting 29.6 ± 1.84 g, with streptococcosis/lactococcosis vaccine at $16 \pm 1.5^\circ\text{C}$, for 60 days. A commercial diet was used as the control. Fish in the first treatment were immunized with streptococcosis/lactococcosis vaccine in bathing route for 1 min. In the second group, the vaccinated trout were also fed diet containing *L. plantarum* (10^8 CFU g⁻¹). In the third treatment, fish were only fed the diet supplemented with *L. plantarum* (10^8 CFU g⁻¹). The results showed that vaccinated trout with or without *L. plantarum* feeding diets significantly decreased heterophils. Meanwhile it enhances serum lysozyme, alternative complement activities, antibody titer, total leukocytes, lymphocytes, and serum biochemical parameters, including ALP, IgM, and total protein levels compared to control groups. Moreover, the highest levels of above mentioned parameters were found in vaccinated fish that fed *L. plantarum*. In addition, the vaccinated fish that fed *L. plantarum* showed significantly elevated cholesterol levels compared to the control group. The results showed that the dietary *L. plantarum* improved the immunity of immunized trout with streptococcosis/lactococcosis vaccine.

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Introduction

Streptococcosis and lactococcosis, gram-positive bacteria, are major systemic bacterial diseases occurring in both wild and cultured fishes (Pridgeon and Klesius, 2012; Soltani et al., 2005). During the examinations of the 108 gram-positive isolates cultured from diseased trout in seven provinces of Iran with major trout production from 2008 to 2009, 49 samples were identified as *S. iniae*, 37 samples were matched *Lactococcus garvieae*, and 22 samples identified as *Streptooccus* sp. (Haghighi Karsidani et al., 2010), suggesting that the trout farms in Iran are severely affected by these diseases. Due to increasing in the resistant bacteria, demands for an effective vaccine have prompted over the past decade (Soltani et al., 2007; Sun et al., 2010; Pridgeon and Klesius, 2011; Sun et al., 2013). Nonetheless, to date the initiative to control these

diseases have focused on the use of antibiotics, including enrofloxacin, oxytetracycline erythromycin and amoxicillin (Austin and Austin, 2007; Evans et al., 2004).

Currently, several experimental Streptococcosis and lactococcosis vaccines in the forms of formalin-killed (Soltani et al., 2007), subunit vaccines (Cheng et al., 2010; Zou et al., 2011), DNA vaccines (Sun et al., 2013), and attenuated live vaccines (Buchanan et al., 2005) are reported. However, the only licensed vaccines against streptococcosis and lactococcosis are bacterins consisting of inactivated whole-cell bacteria (Sommerset et al., 2005). In Iran, a bacterin vaccine is currently available to protect rainbow trout (*Oncorhynchus mykiss*) from streptococcosis/lactococcosis, which has given impressive results (Soltani et al., 2007). Bacterins have also been used to immunize farmed fish in Korea, Australia, Spain

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Table 1. Protocol of experimental groups studied in the present study.

Trial	Immunization regime	Feeding regime	Feeding rate
1	Streptococcosis/lactococcosis vaccine	<i>L. plantarum</i> (1 g/ kg food)	2%
2	Streptococcosis/lactococcosis vaccine	Commercial diet without probiotic	2%
3	Unvaccinated	<i>L. plantarum</i> (1 g/kg food)	2%
4	Unvaccinated	Commercial diet without probiotic	2%

and Chile (Hastein et al., 2005; Sommerset et al., 2005; Austin and Austin, 2007). However, the protectivity of these bacterins in some cases was not completely satisfied (Bachrach et al., 2001; Eyngor et al., 2008).

Recently, the use of probiotic, especially lactic acid bacteria (LAB), as a preferable method that enhances the non-specific immune response of fish has been grown significantly. This improves the prevention and control of various diseases in aquaculture (Cruz et al., 2012). *Lactobacillus plantarum* is a rod-shaped gram-positive bacterium belongs to LAB, and is known to produce plantaricin that is active against certain pathogens (Cebeci and Gurakan, 2003). In aquaculture, the administration of *L. plantarum* induced immune modulation, enhances the growth performance, and increases disease resistance in fishes (Giri et al., 2013, 2014; Son et al., 2009). Nonetheless, there is a lack of information regarding *L. plantarum* effects on vaccinated fish. Therefore, the present study was carried out to explore the influence of probiotic, *L. plantarum* on some serum biochemical and some immune parameters of vaccinated rainbow trout against streptococcosis/lactococcosis.

Materials and Methods

Probiotic: A commercial probiotic *L. plantarum* (kc426951) isolated from the intestinal tract of rainbow trout was used in this study. The fish fed with commercial diet (Faradaneh, Iran) supplemented with *L. plantarum* as the probiotic cells suspension that has added in the feed as 1 g (10^8 cells g^{-1})/ kg feed.

Fish culture and experimental design: Juveniles of rainbow trout weighting 29.6 ± 1.84 g were obtained from a local hatchery in Arak, Iran. The fish were

acclimatized for two weeks in the laboratory condition in a 1000-L tank with a flow-through system (7 L min^{-1}) at $16 \pm 1.5^\circ\text{C}$ and were fed a commercial diet (Faradaneh, Iran). After checking the health status, acclimatized fish were randomly distributed into twelve 1000-L tanks, four groups each in three replicate with 80 fish per replicate.

Two groups of fish were immunized with streptococcosis/lactococcosis vaccine (Soltani et al., 2007) in bathing route for 1 min under aeration. The other two groups were considered as unvaccinated fish. One of each vaccinated and unvaccinated group were fed with diet containing *L. plantarum* (1 g/kg food), while the other two groups were fed without *L. plantarum*. Protocol of experimental groups studied is given in Table 1.

On 12, 24, 36, 48 and 60 days of the experiment, nine fish from each replication were anesthetized with clove powder solution (200 mg l^{-1}), and blood was collected from caudal vein puncture. Then, serum biochemical and some immune parameters analysis were carried out on blood samples.

During the feeding trial, the fish were fed three times a day (09:00, 12:00 and 15:00) at rate of 2% of body weight. The feeding rate was corrected every 12 days following a 24 hrs starvation period and batch weighing. The experiment was carried out for 60 days. Water quality parameters including water temperature, pH and dissolved oxygen were monitored daily and were maintained at $16 \pm 1.5^\circ\text{C}$, 7.5 ± 0.5 and 8 ± 0.4 mg/L, during the experimental period, respectively.

Serum biochemical parameters assay: Serum biochemical analysis, including alkaline phosphatase (ALP), cholesterol, total protein, and albumin levels were measured by a commercial kit according to the manufacturer protocol (Parsazmon

Table 2. Serum biochemical parameters in rainbow trout (*Oncorhynchus mykiss*) fed with probiotic (*L. plantarum*) and immunized with streptococcus/lactococosis vaccine.

Parameter/Treatment	Time elapsed (Days)				
	12	24	36	48	60
ALP (U dL⁻¹)					
Control	210.7 ± 46 ^a	220.7 ± 32 ^a	249.3 ± 35 ^a	287.7 ± 33 ^a	320.7±39 ^a
P	246 ± 38 ^a	287.3 ± 32 ^a	353 ± 29 ^b	403.7 ± 37 ^b	453.7 ± 31 ^b ^c
V	383.67±33 ^b	491±48 ^b	567±49 ^c	490.67±54 ^c	437.67±38 ^b
V+P	384.33±43 ^b	570±47 ^c	607±42 ^c	584.67±31 ^d	514.33±47 ^b
Total Protein (g dL⁻¹)					
Control	2.58±0.3 ^a	2.98±0.22 ^a	3.07±0.26 ^a	3.49±0.15 ^a	3.25±0.2 ^a
P	2.81±0.23 ^a	3.14±0.21 ^a	3.27±0.21 ^a	3.44±0.22 ^a	3.51±0.27 ^{ab}
V	3.88±0.34 ^b	4.26±0.17 ^b	3.95±0.16 ^b	3.84±0.18 ^b	3.92±0.23 ^b
V+P	3.93±0.28 ^b	4.35±0.25 ^b	4.21±0.22 ^b	4.05±0.26 ^b	4.1±0.21 ^b
Albumin (g dL⁻¹)					
Control	1.5 ± 0.18 ^a	1.61±0.16 ^a	1.46±0.18 ^a	1.5±0.09 ^a	1.44±0.08 ^a
P	1.52 ± 0.23 ^a	1.65±0.15 ^a	1.4±0.1 ^a	1.63±0.06 ^a	1.79±0.1 ^b
V	1.47 ± 0.17 ^a	1.75±0.11 ^a	1.62±0.1 ^a	1.58±0.08 ^a	1.49±0.11 ^a
V+P	1.56 ± 0.16 ^a	1.54±0.26 ^a	1.69±0.13 ^a	1.62±0.11 ^a	1.43±0.15 ^a
IgM (mg mL⁻¹)					
Control	1.68±0.28 ^a	1.95±0.46 ^a	2.02±0.4 ^a	2.29±0.37 ^a	2.25±0.33 ^a
P	1.79±0.33 ^a	1.96±0.3 ^a	2±0.36 ^a	2.21±0.46 ^a	2.7±0.25 ^{ab}
V	2.77±0.57 ^b	3.68±0.31 ^b	4.08±0.36 ^b	3.53±0.54 ^b	3.31±0.46 ^b
V+P	2.68±0.46 ^b	3.96±0.47 ^b	4.29±0.41 ^b	3.8±0.29 ^b	3.57±0.36 ^b
Cholesterol (mg dL⁻¹)					
Control	215±40 ^a	292±10 ^a	262±14 ^a	295±14 ^a	279±20 ^{ab}
P	207±36 ^a	286±17 ^a	218±25 ^a	237±13 ^a	217±24 ^a
V	213±30 ^a	295±16 ^a	229±10 ^a	268±31 ^a	245±36 ^a
V+P	233±22 ^a	293±9 ^a	220±19 ^a	271±22 ^a	311±11 ^b

P: fish fed with probiotic (*L. plantarum*), V: fish immunized with streptococcus/lactococosis vaccine, P+V: fish fed with probiotic (*L. plantarum*) and immunized with streptococcus/lactococosis vaccine, Control: fish fed with commercial diet. Values (Mean±SE, n=27) containing different superscripts in the same row denotes significant difference between the treatments ($P<0.05$).

Co. Iran). Serum total IgM levels were measured with an Enzyme-linked Immunosorbent Assay (ELISA) using a commercial kit (Cusabio, Wuhan, Hubei, China), as described by Sun et al. (2010).

Immune parameters

Leukocytes: To assess leukocytes, appropriate blood smears were prepared from blood samples which were obtained on the 12, 24, 36, 48 and 60th days of the experiment. Smears were then air-dried, fixed in 96% ethanol for 30 min and stained with Giemsa for 30 min. The smears were examined for total leukocyte counts, lymphocytes, and heterophils under a compound microscope (Klontz, 1994).

Serum lysozyme activity: Lysozyme activity was measured based on Ellis (1990) with a slight modifications carried out.

Alternative complement activity (ACH₅₀):

Alternative complement activity assays was carried out following the procedure of Yano (1992) using rabbit red blood cells (RaRBC). The calculation of the complement activity was carried out using below equation:

$$\text{ACH}_{50} \text{ value (units mL}^{-1}\text{)} = 1/K \times (\text{reciprocal of the serum dilution}) \times 0.5$$

Where K is the amount of serum (mL) giving 50% lyses and 0.5 is the correction factor since this assay is performed on the half scale of the original method.

Agglutination antibody titer: Agglutinating antibody titer to *S. iniae* were measured using the micro-agglutination test (Roberson, 1990).

Statistical analysis: The mean and SEM were determined for 27 fish per each treatment. All the

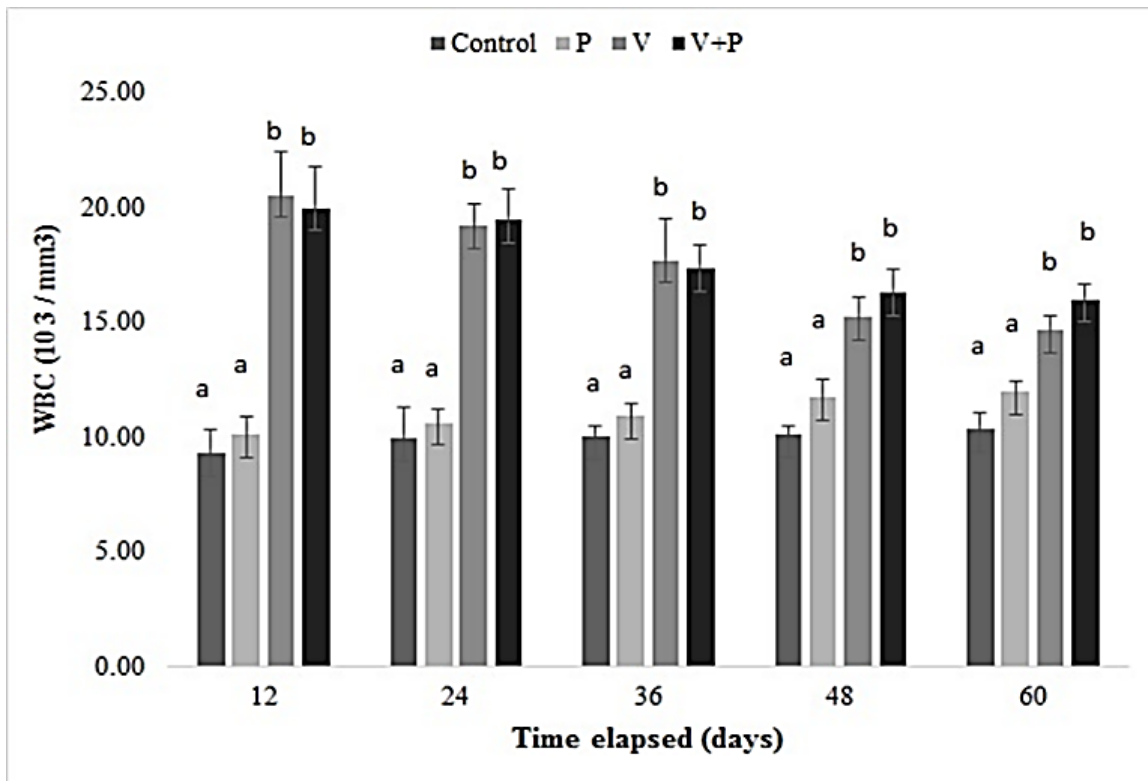


Figure 1. Total leukocyte counts in rainbow trout fed with probiotic (*L. plantarum*) and immunized with streptococcus/lactococosis vaccine at $16 \pm 1.5^\circ\text{C}$, for 60 days (P: fish fed with probiotic (*L. plantarum*), V: fish immunized with streptococcus/lactococosis vaccine, P+V: fish fed with probiotic (*L. plantarum*) and immunized with streptococcus/lactococosis vaccine, Control: fish fed with commercial diet). Values (Mean \pm SE, n=27) containing different superscripts in the same row denotes significant difference between the treatments ($P < 0.05$).

data were analyzed using one-way ANOVA by SPSS package (SPSS 1998) at the 0.05 significance level.

Results

No mortality was observed during the experiment. The water quality of each group did not change during the experiment due to daily renewing of water.

Serum biochemical parameters: The results of serum biochemical parameters are presented in Table 2. The alkaline phosphatase (ALP) levels of all treatments were significantly higher ($P < 0.05$) than that of the control group after 36, 48 and 60 days. Moreover, this value was significantly higher in vaccinated trout with/without *L. plantarum* after 12 and 24 days of the experiment. However, its levels changed differently only in vaccinated fish fed probiotic on day 12 ($P < 0.05$).

After 60 days of feeding, the vaccinated fish fed the diet supplemented with *L. plantarum* showed significantly higher cholesterol than the control

group ($P < 0.05$). The total protein and IgM levels of vaccinated trout with/without *L. plantarum* were significantly higher than those of the control group on all sampling day of the experiment ($P < 0.05$). However, no significant difference was found in albumin value in treatment groups compared to the control group ($P > 0.05$).

Immune parameters

Leukocytes: Total leukocyte counts (Fig. 1) and lymphocyte values (Fig. 2A) in immunized trout with streptococcosis/lactococosis vaccine with/without feeding diets containing *L. plantarum* after 12, 24, 36, 48 and 60 days were significantly higher than the control group ($P < 0.05$). In contrast, the level of heterophil (Fig. 2B) was lower in the treated groups compared to the control group ($P < 0.05$). However, no significant changes were found in total leukocyte counts, lymphocytes and heterophil levels of fish fed the diet supplementation of *L. plantarum* ($P > 0.05$).

Lysozyme activity: The results of serum lysozyme

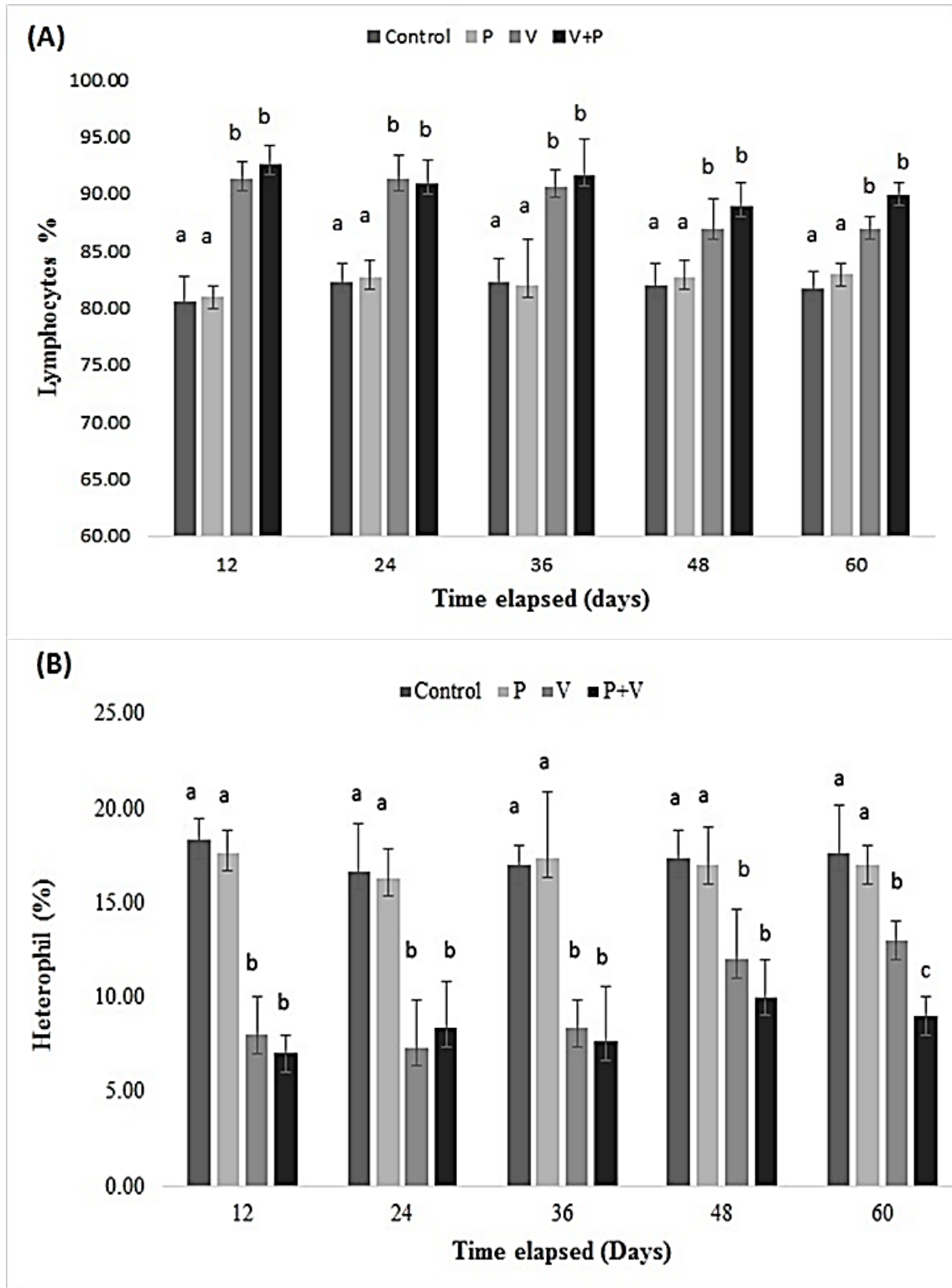


Figure 2. Leukocyte differential count (A: Lymphocyte, B: Heterophil) in rainbow trout fed with probiotic (*L. plantarum*) and immunized with streptococcus/lactococosis vaccine at $16\pm 1.5^{\circ}\text{C}$, for 60 days (P: fish fed with probiotic (*L. plantarum*), V: fish immunized with streptococcus/lactococosis vaccine, P+V: fish fed with probiotic (*L. plantarum*) and immunized with streptococcus/lactococosis vaccine, Control: fish fed with commercial diet). Values (Mean \pm SE, n=27) containing different superscripts in the same row denotes significant difference between the treatments ($P<0.05$).

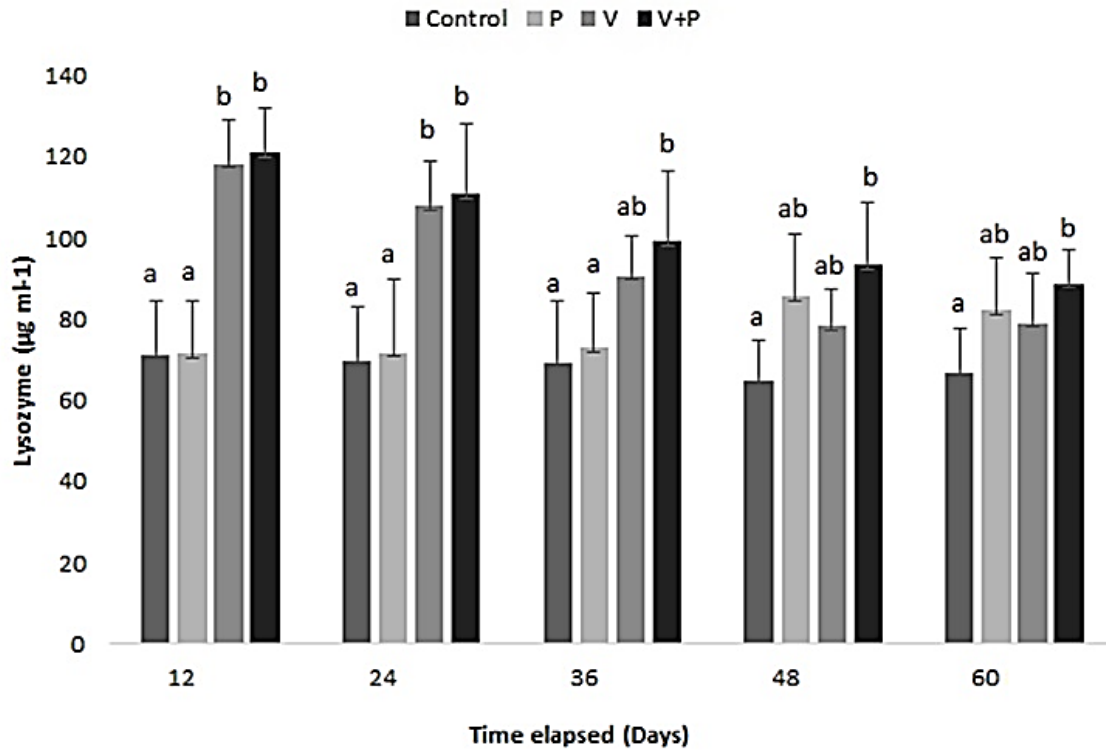


Figure 3. Lysozyme levels in rainbow trout fed with probiotic (*L. plantarum*) and immunized with streptococcus/lactococcosis vaccine at $16 \pm 1.5^\circ\text{C}$, for 60 days (P: fish fed with probiotic (*L. plantarum*), V: fish immunized with streptococcus/lactococcosis vaccine, P+V: fish fed with probiotic (*L. plantarum*) and immunized with streptococcus/lactococcosis vaccine, Control: fish fed with commercial diet). Values (Mean \pm SE, n=27) containing different superscripts in the same row denotes significant difference between the treatments ($P < 0.05$).

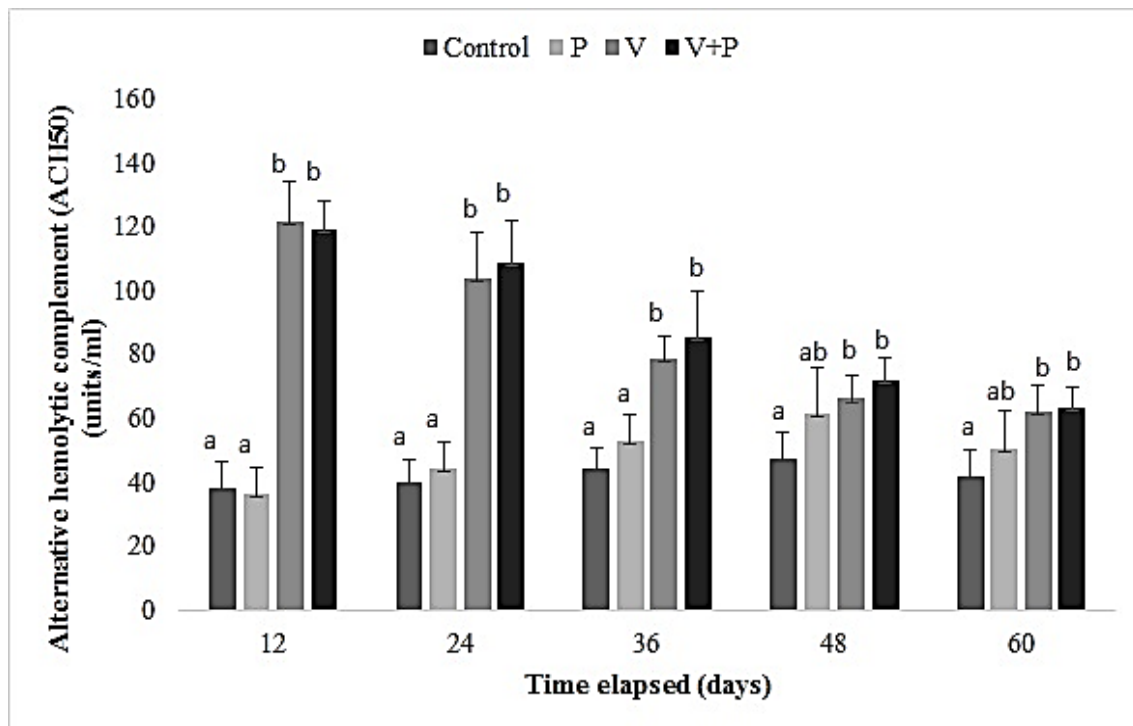


Figure 4. ACH₅₀ values in rainbow trout fed with probiotic (*L. plantarum*) and immunized with streptococcus/lactococcosis vaccine at $16 \pm 1.5^\circ\text{C}$, for 60 days (P: fish fed with probiotic (*L. plantarum*), V: fish immunized with streptococcus / lactococcosis vaccine, P+V: fish fed with probiotic (*L. plantarum*) and immunized with streptococcus/lactococcosis vaccine, Control: fish fed with commercial diet). Values (Mean \pm SE, n=27) containing different superscripts in the same row denotes significant difference between the treatments ($P < 0.05$).

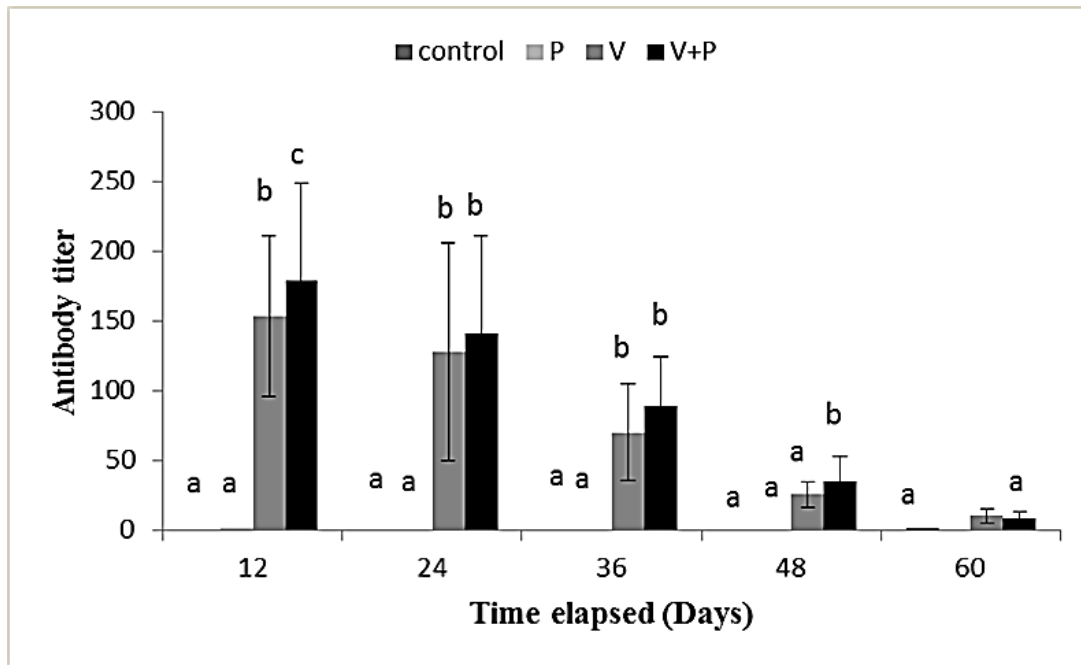


Figure 5. Agglutination antibody titers in rainbow trout fed with probiotic (*L. plantarum*) and immunized with streptococcus/lactococcosis vaccine at $16 \pm 1.5^\circ\text{C}$, for 60 days (P: fish fed with probiotic (*L. plantarum*), V: fish immunized with streptococcus/lactococcosis vaccine, P+V: fish fed with probiotic (*L. plantarum*) and immunized with streptococcus/lactococcosis vaccine, Control: fish fed with commercial diet). Values (Mean \pm SE, n=27) containing different superscripts in the same row denotes significant difference between the treatments ($P < 0.05$).

activity are shown in Figure 3. Lysozyme activity of vaccinated trout fed with *L. plantarum* was significantly higher ($P < 0.05$) than the control group after 60 days of feeding. Moreover, its value increased significantly for 12 and 24 days of the experiment in vaccinated trout without feeding dietary *L. plantarum* ($P < 0.05$). Nonetheless, no significance change was observed in unvaccinated fish that was only fed probiotic ($P > 0.05$).

Alternative complement pathway activity (ACH₅₀):

The results of ACH₅₀ are shown in Figure 4. The serum ACH₅₀ of both vaccinated groups with/without feeding *L. plantarum* diet was significantly higher ($P < 0.05$) than the control group after 12, 24, 36, 48 and 60 days of feeding. However, no significant changes were found in ACH₅₀ in fish fed the diet supplementation of *L. plantarum* ($P > 0.05$).

Antibody titer: The results of antibody titer are shown in Figure 5. Antibody titer to *S. iniae* in both of vaccinated groups with/without *L. plantarum* feeding was gradually decreased from day 12 to 60 days after the vaccination but it was significantly higher than control one up to 48 days of feeding. The highest antibody titer was found in vaccinated trout

that fed diet *L. plantarum* with no significantly different with vaccinated fish fed with probiotic; the value was significantly different from the other groups ($P < 0.05$).

Discussion

The probiotics have been recognized to function as immune-modulators in finfish which is often through stimulation of innate and cellular immunity, including enhanced phagocytic, lysozyme, respiratory burst, cytotoxicity, complement activity, superoxide dismutase, increased numbers of leukocytes, erythrocytes, monocytes and lymphocytes, migration of neutrophils, neutrophil adherence, antiprotease and peroxidase activities, and plasma bactericidal activity (Newaj-Fyzul and Austin, 2015). In addition, there may be increases in serum bacterial agglutination antibody titer (Ridha and Azad, 2012), albumin (humoral immunity) and total IgM levels (Sharifuzzaman and Austin, 2010a, 2010b). Nonetheless, various probiotics may show different type of immune response.

In the present study, serum biochemical parameters, including ALP, cholesterol, serum total

protein and total IgM values of vaccinated trout fed the probiotic were significantly higher compared to the control group. The ALP is associated with the absorption of lipid, glucose, calcium and inorganic phosphate (Eguchi, 1995). In this study, the alkaline phosphatase (ALP) levels of all treatment groups were significantly higher than the control group. Increased phosphatase activity indicates a higher breakdown of the energy reserve, which may be utilized for the enhancement of growth or immunity (Ghosh et al., 2008), as the result of vaccination and/or fed *L. plantarum* diet. Furthermore, higher levels of cholesterol were shown in vaccinated trout fed *L. plantarum* diet, after 60 days of feeding, which may be due to the increased activity of liver enzymes like the ALP. This indicates the disorders of lipid and lipoprotein metabolism (Allen et al., 2005). In the present study, the highest levels of serum IgM were observed in vaccinated trout fed with *L. plantarum* after 36 days of feeding. In spite of the slight decrease, its levels increased significantly more than the control group after 60 days of feeding. Nonetheless, serum total IgM levels in unvaccinated trout fed *L. plantarum* diet did not show a significant change compared to control group. In contrast, dietary supplement-ation of *Bacillus* in grouper until 30 days (Sun et al., 2010), and in rainbow trout fed dietary *L. rhamnosus* (JCM 1136) up to 20 days (Panigrahi et al., 2005) have shown an increase in IgM levels trend of feeding, and thereafter a dropping pattern prevailed.

The total protein levels of vaccinated trout with/without feeding diets containing *L. plantarum* were significantly higher than those of the control groups on all sampling days of the experiment. The increase in serum total protein content might be due to an increase in the leukocytes, which is a major source of serum protein production, including complement factors, lysozyme, and bactericidal peptides (Misra et al., 2006). This is supported by increase in leukocytes value in the both vaccinated groups. Probably, the increase in the leukocyte count might have resulted in the enhancement of the non-specific immunity. Besides, the total leukocyte

count, lymphocytes increased in both mentioned groups. Meanwhile, heterophil were significantly decreased compared to the control group, similar to pervious study by Soltani et al. (2007) who evaluated these parameters in immunized trout with streptococcosis/lactococcosis vaccine.

Significant higher serum lysozyme activity and alternative complement activity (ACP₅₀), and serum antibody titers were shown in both vaccinated groups with/without feeding dietary *L. plantarum*, compared to the control groups. While these parameters did not increase in fish that were fed only probiotic, *L. plantarum*, in lysozyme and ACH₅₀ activity simulation of *L. plantarum* has been previously showed in different studies (Son et al., 2009; Giri et al., 2013). The discrepancy of these findings may be attributable to the difference in probiotic doses and the feeding duration. Beside, vaccines have been shown to increase serum lysozyme activities, alternative complement activity and antibody titers in rainbow trout (Kim and Austin, 2006; Soltani et al., 2007), which suggests an enhancement of these parameters due to using vaccine.

In conclusion, the present results demonstrated that the administration of dietary *L. plantarum* (1 g/kg food) in vaccinated trout against streptococcosis /lactococcosis can induce some of the specific and non-specific immune responses. This appears to be obtained by increasing antibody titer, lysozyme activity, alternative complement activity and some serum biochemical parameters such as IgM levels. To our knowledge, this is the first study that investigated a positive probiotic dietary in vaccinated trout.

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