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Effect of the probiotic, *Lactobacillus plantarum* on growth performance and haematological indices of rainbow trout (*Oncorhynchus mykiss*) immunized with bivalent streptococcosis / lactococcosis vaccine

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Abstract

The present study evaluated the effect of *Lactobacillus plantarum* as a probiotic on the growth performance and haematological parameters of juvenile rainbow trout (*Oncorhynchus mykiss*) weighing 29.6±1.8 g immunized with streptococcus/lactococcus bivalent vaccine for 60 days at 16±1.5ºC. Fish were randomly allocated to 4 equal groups: probiotic (g kg^{-1} feed equal to 10^8 cells g^{-1}) supplemented diet group (group P), vaccinated fish fed with a normal diet (group V), vaccinated fish fed with probiotic (group P+V) and unvaccinated fish fed normal diet (group C). Results showed that weight gain, final weight, condition factor and thermal-unit growth coefficient were improved in P+V group compared with other groups but was significantly different from groups V and C ($p<0.05$). Also, feed conversion ratio in the P+V group was lower than other trails ($p<0.05$). In addition, white blood cell count (WBC) in groups V and P+V were significantly higher than in both P and C groups ($p<0.05$). Also, PCV was significantly increased ($p<0.05$) in both P and P+V groups compared to group V and the control group. No significant difference ($p>0.05$) was found in RBC, Hb, MCH, MCHC and MCV levels among the different experimental groups. These data show that application of *L. plantarum* could be a benefit of vaccinated trout, enhancing fish growth and haematological parameters.

Keywords: *Lactobacillus plantarum*, probiotic, trout, haematology, growth, vaccine, streptococcosis/lactococcosis

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Introduction

Aquaculture is an important food producing sector, but is faced with significant constraints due to several infectious diseases including streptococcosis and lactococcosis (Austin and Austin, 2007; Bostock *et al*., 2010). These bacterial infections are widely spread in many regions, including Iranian trout (*O. mykiss*) farms causing huge economic losses (Soltani *et al*., 2005; Soltani *et al*., 2008; Erfanmanesh *et al*., 2012). To date, control of these diseases has been focused upon the use of antibiotics, and more recently on vaccinations (Evans *et al*., 2004; Soltani *et al*., 2007; Sun *et al*., 2010). Application of antibiotics is the common way to treat bacterial infections in aquaculture however, this leads to overuse and gives rise to resistant strains (Karunasagar *et al*., 1994; Akinbowale *et al*., 2006). A local vaccine is currently available to protect Iranian rainbow trout farms with encouraging results so far.

Besides vaccination, the use of probiotics, in preference to antibiotics, is increasingly gaining acceptance for the control of pathogens in aquaculture (Sheehan *et al*., 2009; Raissy *et al*., 2018). Probiotics have been associated with many useful effects on growth performance (Giannenas *et al*., 2015; Sivani *et al*., 2016), disease resistance by enhancing innate immunity (Irianto and Austin, 2002; Cha *et al*., 2013; Safari *et al*., 2016), reduction in pathogenic microbiotia of fish gut (Abid *et al*., 2013), and with a consequence in a less chemotherapy (Irianto and Austin, 2002; Azad and Al-Marzouk, 2008).

Several studies have shown lactic acid bacteria (LAB) such as *Lactobacillus plantarum*, especially from the gastrointestinal tract of fish, to be beneficial for their probiotic properties in aquaculture (Ringo and Gatesoupe, 1998; Ali and Sahu, 2002; Balcázar *et al*., 2008; Harikrishnan *et al*., 2010; Giri *et al*., 2013).

Application of *L. plantarum* as probiotic illustrated to increase the disease resistance, non-specific immune defence and growth performance in fish (Son *et al*., 2009; Giri *et al*., 2014; Dawood *et al*., 2015). However, only a few data available on the effect of probiotics on growth and immune-physilogy of vaccinated fish. Tilapia vaccinated with an *A. hydrophila* bacterin and fed *Lactobacillus* at various concentrations significantly activated neutrophils, and lymphocytes compared to vaccine only group (Venkatalakshmi & Ebanasar 2015). Oral application of the mixture of *Lactobacillus acidophilus*, *Bacillus subtilis*, *Saccharomyces cervisiae* and *Aspergillus oryzae* to tilapia vaccinated against *A. hydrophila* significantly increased survival from the challenge infection (Aly et al. 2016). Oral use of *L. plantarum* in trout bath-vaccinated against *Yersinia ruckeri* elevated some immunological responses and growth of vaccinated fish (Soltani et al. 2017a, b). The present study aimed to explore the effects of *L. plantarum* as probiotic on haematological parameters and growth performance in trout immunized with streptococcus/lactococcosis bivalent vaccine.

Materials and methods

Probioitc

L. plantarum (kc426951) previously isolated from the intestinal tract of rainbow trout was used for this study. The probiotic cell suspension was added to commercial fish diet (Faradaneh, Shahrekord, Iran) at a concentration of 1g (10^8cells g^{-1}) kg^{-1} feed.

Fish culture and experimental design

Juvenile rainbow trout (*O. mykiss*) weighing 29.6±1.8 g obtained from a local hatchery in Arak province, Iran were allowed to acclimatize for 14 days in a 5000-L tank with a flow-through system (7 L min^{-1}), and were fed a commercial diet (Faradaneh, Shahrekord, Iran). Fish were then randomly distributed into twelve 1000-L tanks with 80 fish per tank, four treatments each in three replicates.

Four groups were included: fish fed with the probiotic at g kg^{-1} feed equal to 10^8 cells g^{-1} (group P), vaccinated fish fed with the probiotic (group P+V), vaccinated fish fed with a normal diet (group V) and unvaccinated fish fed normal diet (group C). Bivalent vaccine against streptococcosis/lactococcosis (formalin inactivated cells at $1x10^9$ cells ml^{-1} of each *Streptococcus iniae* and *Lactococcus garviae* strains) was used. Vaccination was preformed using the bath method for 1 min under good aeration. The protocol of experimental groups is given in Table 1.

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

Immunization regime	Feeding regime	Feeding rate
Vaccinated fish	Probiotic (1g kg^{-1} food)	2%
Vaccinated fish	Normal diet without probiotic	2%
Unvaccinated fish	Probiotic (1g kg^{-1} food)	2%
Unvaccinated fish	Normal diet without probiotic	2%

During the feeding trial, the fish were fed three times a day (09:00, 12:00 and 15:00 hours) at the rate of 2% of body weight. The feeding rate was corrected every 12 days. The water quality parameters including pH, dissolved oxygen, water temperature, NO_2 and NH_3 were monitored daily and maintained at 7.5±0.5, 8±0.4 mg L^{-1}, 16±1.5 ºC, <0.1 mg L^{-1} and 0.01 mg L^{-1}, respectively during the 60 days of the experimental period.

Growth performance

At the end of the experiment the weight and size of individual fish (n=9 per replicate) in the tanks of each treatment group (n=27 per group) were measured, and feed utilization and growth parameters were calculated using the following formulae:

Condition factor=weight/(length)^3×100

Feed conversion ratio (FCR)=food consumption (g)/weight gain (g)

Weight gain (g)=final weight–initial weight

Thermal-unit growth coefficient (TGC) = $(W_2^{(1/3)} – W_1^{(1/3)})/(T \times \Delta t) \times 1000$

Where W_1 is the group average initial weight (g), W_2 is the group average weight at time t (g), T is the water temperature (^{o}C) and Δt is the duration of the experiment (days).

Haematological assay

On days 12, 24, 36, 48 and 60 of the experiment, nine fish from each group were anesthetized with clove oil (200 mg L^{-1}), and blood was collected from the caudal vein. Haematological parameters including white blood cells (WBCs), erythrocyte count (RBC), packed cell volume (PCV), haemoglobin (Hb), mean erythrocyte haemoglobin (MCH), mean erythrocyte haemoglobin concentration (MCHC) and mean erythrocyte cell volumes (MCV) were measured (Svobodova *et al*., 1991).

Statistical analysis

The mean and SEM were determined, and the data were analysed using one-way ANOVA using SPSS package (SPSS 1998) at the 0.05 significance level.

Results

Growth performance

The results of feed utilization and growth performance parameters are given in Table 2. All groups showed above 97 % survival rates (p<0.05). The results revealed that growth performance parameters including weight gain, final weight, TGC and FCR were significantly (p<0.05) improved in all treatment groups compared to the control, and the highest values of final weight (156.24±2.29 g), weight gain (126.74±2.29 g) and TGC (0.201±0.003) plus the lowest level of FCR (0.96±0.02) were obtained in Group P+V. In addition, Group P+V significantly enhanced condition factor compared to the other groups (1.1±0.05) (p<0.05).

Haematological parameters

Results of WBC are given in Fig. 1**.** On days 12, 24, 36, 48 and 60, WBC levels in groups V and P+V, were significantly higher than in the other two groups. However, no significant change was found in group P (Fig. 1).

Also, results of RBC are shown in Fig. 2. On days 12 and 48, RBC level in group P+V was significantly higher than C group (Control) (p<0.05), (Fig. 2). No significant differences were seen among other trails during the experiment (p>0.05).

Table 2: Mean ± SD of growth performance and feed utilization parameters in trout immunized with streptococcosis/lactococosis vaccine and fed *Lactobacillus plantarum* for 2 months at 16±1.5 ºC.

Growth factor	C (control)	P	V	V+ P
Initial weight (g)	29.5 ±1.98	29.54±1.86	29.88±1.81	29.50±1.76
Final weight (g)	140.22±2.91^{a}	152.83±2.59^{bc}	148.47±2.67^{b}	156.24±2.29^{c}
Weight gain (g)	110.72±2.91^{a}	123.29±2.59^{bc}	118.59±2.67^{b}	126.74±2.29^{c}
Feed (g fish^{-1})	127.67±7.02	120±5.29	123.17±3.88	121.33±5.13
FCR	1.15±0.03^{a}	0.97±0.03^{by}	1.04±0.03^{b}	0.96±0.02^{c}
Initial size (cm)	14.50±0.22	14.40±0.2	14.55±0.28	14.40±0.23
Final size (cm)	23.99±0.18^{a}	24.13±0.34^{a}	24.05±0.45^{a}	24.23±0.48^{a}
Condition factor	1.02±0.02^{a}	1.09±0.03^{ab}	1.07±0.05^{ab}	1.1±0.05^{b}
TGC	0.185±0.003^{a}	0.198±0.003^{bc}	0.192±0.003^{b}	0.201±0.003^{c}

FCR: Feed conversion ratio, TGC: Thermal-unit growth coefficient, P: fish fed with probiotic (*L. plantarum*), V: fish immunized with streptococcosis/lactococosis vaccine, P+V: fish fed with probiotic (*L. plantarum*) and immunized with streptococcosis/lactococosis vaccine, Control: fish fed with normal diet. Values (n=27) within the same row with different superscripts denotes significant differences (p<0.05).

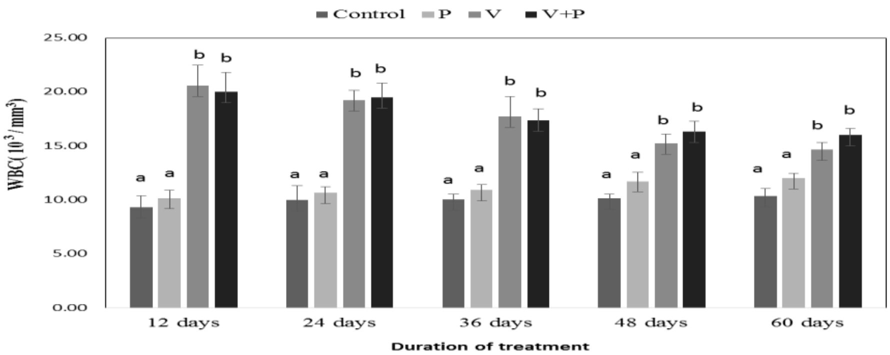

Figure 1: White blood cells (WBC) in vaccinated rainbow trout with streptococcosis / lactococcosis vaccine and fed with probiotic (*Lactobacillus plantarum*) for 2 months at 16±1.5ºC. P: unvaccinated fish fed with the probiotic (*L. plantarum*), V: fish immunized with streptococcosis / lactococcosis vaccine and fed with a normal diet, P+V: fish immunized with streptococcosis / lactococcosis vaccine and fed with the probiotic supplemented in diet, Control: unvaccinated fish fed with normal diet. Values (n=27) within the same row with different superscripts denotes significant differences (*p*<0.05).

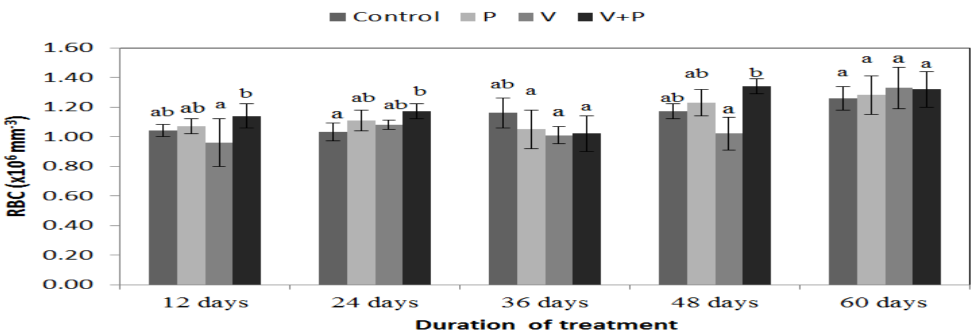

Figure 2: Red blood cells (RBC) in vaccinated rainbow trout with streptococcosis / lactococcosis vaccine and fed with probiotic (*Lactobacillus plantarum*) for 2 months at 16±1.5ºC. P: unvaccinated fish fed with the probiotic (*L. plantarum*), V: fish immunized with streptococcosis / lactococcosis vaccine and fed with a normal diet, P+V: fish immunized with streptococcosis / lactococcosis vaccine and fed with the probiotic supplemented in diet, Control: unvaccinated fish fed with normal diet. Values (n=27) within the same row with different superscripts denotes significant differences (*p*<0.05).

In addition, group P+V showed a significant increase in Hb level at 36 days ($p<0.05$). Results also showed that fish in groups P and P+V had significantly higher levels of PCV on days 24 and 48 ($p<0.05$). No significant differences were found ($p>0.05$) in MCH, MCHC and MCV levels among the trails during 2 month experiment (Table 2).

Table 3: The mean±SD of derived hematological parameters in trout immunized with streptococcus / lactococosis vaccine and fed a probiotic (*Lactobacillus plantarum*) for 2 months at16±1.5 ºC.

Treatments	Hb (g dL^{-1})	PCV (%)	MCHC (g dL^{-1})	MCH (pg)	MCV (fl)
12 days					
Control	5.69±0.3^a	32±1^a	17.78±0.4^a	54.75±2.46^a	307.79±8.16^a
P	5.86±0.45^a	33.33±1.53^a	17.56±056^a	54.58±2.92^a	310.67±9.29^a
V	5.38±0.6^a	32.67±3.06^a	16.46±0.38^a	56.48±5.22^a	343.15±31.74^a
V+ P	6.45±0.84^a	36±3^a	17.87±0.87^a	56.31±4.38^a	314.79±9.97^a
24 days					
Control	5.19±0.15^a	31.33±0.58^a	16.57±0.39^a	50.66±2.66^a	305.61±11.43^a
P	5.75±0.4^a	35.33±2.08^b	16.28±0.66^a	52±5.46^a	318.81±21.9^a
V	5.84±0.32^a	33.33±0.58^a	17.51±0.66^a	54.31±4.21^a	309.79±12.46^a
V + P	6.2±0.32^a	36±1^b	17.21±0.75^a	52.82±1.75^a	306.94±5.09^a
36 days					
Control	6.99±0.17^{ab}	33±2^a	21.24±1.8^a	60.57±6.36^a	285.66±6.82^a
P	7.09±0.38^{ab}	33.67±2.52^a	21.16±2.08^a	68.34±5.88^a	321.95±16.02^a
V	6.5±0.21^a	34.67±1.53^a	19.36±1.51^a	64.37±5.42^a	323.47±10.25^a
V+ P	7.31±0.35^b	32.33±2.08^a	22.64±0.71^a	70.21±6.46^a	318.6±19.7^a
48 days					
Control	7.25±0.46^a	36.33±1.53^a	19.95±0.46^a	62.14±1.44^a	311.48±5.58^a
P	8.06±0.34^a	42.67±1.53^b	18.92±1.13^a	65.66±2.11^a	348.25±30.75^a
V	7.1±0.64^a	34.67±2.08^a	20.45±0.64^a	69.45±1.57^a	339.91±15.87^a
V + P	8.42±0.95^a	43±1^b	18.73±2.24^a	62.91±5.12^a	337.01±17^a
60 days					
Control	7.62±0.25^a	41.33±1.53^a	18.47±1.19^a	60.87±5.29^a	329.23±7.72^a
P	7.78±0.24^a	43.33±1.53^a	17.96±1.19^a	60.9±4.24^a	339.14±23.06^a
V	7.34±0.37^a	43.33±1.53^a	17.07±0.49^a	55.28±3.16^a	324.21±25.35^a
V + P	8.02±0.42^a	43.57±1.53^a	18.35±0.36^a	60.74±2.62^a	331.18±19.66^a

P: unvaccinated fish fed with the probiotic (*L. plantarum*), V: fish immunized with streptococcosis /lactococcosis vaccine and fed a normal diet, P+V: fish immunized with streptococcosis/lactococcosis vaccine and fed with the probiotic supplemented in diet, Control: unvaccinated fish fed a normal diet. Values (n=27) within the same row with different superscripts denotes significant differences ($p<0.05$). Values (n=27) within the same row with different superscripts denotes significant differences ($p<0.05$).

Discussion

In vertebrates, it has been shown that maintaining and improving an active immune system can be energetically too costly as it is necessary to adjustment physiological activities (Fair *et al*., 1999; Pilorz *et al*., 2005). It has been shown that increases in immune response cause adverse effects on biological functions, possibly through an increase in metabolic rate (Pilorz *et al.*, 2005). Moreover, Ackerman *et al*. (2000) reported that administration of a monovalent, adjuvanted *Aeromonas* *salmonicida* vaccine increases the metabolic rate of juvenile trout. Considering the negative correlation between growth performance and metabolic rate, probiotics may help to compensate this deficiency in vaccinated fish. While there have been some individual works investigating the effects of vaccination and probiotic diets on the immune response and growth performance of fish, our study evaluated the dietary administration of probiotic *L. plantarum* alone and concurrently with streptococcosis/lactococcosis vaccine on haematological and growth performance of rainbow trout. The results of the present study showed that both the streptococcus/lactococcosis vaccine and dietary supplementation with *L. plantarum* (10^8 CFU g^{-1}) improved the feed utilization and growth performance parameters in trout. This might be in part due to an attribution of the increased digestibility coefficient of the diet (Dawood *et al*., 2015). Also, modulating the digestive physiology of fish by enhancing intestinal enzymes activity can improve the growth of fish fed a probiotic as mentioned by several workers (Yanbo and Zirong, 2006; Lazado *et al*., 2012; Sun *et al*., 2012). For example, use of diets supplemented with *L. plantarum* improved feed efficiency and growth in different fish species (Son *et al*., 2009; Jatobá *et al*., 2011; Parthasarathy and Ravi, 2011; Giri *et al*., 2013). In the present study the growth performance including weight gain, final weight, and TGC were significantly ($p<0.05$) improved in all treated groups compared with the control. However, the highest values of final weight (156.24±2.29 g), weight gain (126.74±2.29 g) and TGC (0.201±0.003) were obtained in vaccinated fish fed with the probiotic (group V+P). Also, the lowest level of FCR (0.96±0.02) were seen in this group. The reason why the application of the probiotic can provide such better growth conditions in vaccinated fish is not clear and warrants further studies. However, it may in part be due to the enchantment of some non-specific factors, e.g. lysozyme, complement component, phagocytosis in the vaccinated fish that can provide a better condition for fish physiology to challenge with the stress condition during the rearing period. This is supported by a higher level of immunocompetent cell population (WBC) in V+P group through the experiment (Fig. 1). Neutrophils and lymphocytes were increased in the vaccinated tilapia fed with *Lactobacillus* (Venkatalakshmi & Ebanasar 2015). Also, our results showed that the weight gain, final weight TGC in P group were higher than V group and the control (Table 1). This is because when a fish population is immunized with an antigen/vaccine, the growth condition can be negatively influenced at least for a short time (Ward *et al*., 1985; Midtlying and Lillehaug, 1998; Koskela *et al*., 2004) because of a force on fish immune-physiological activities to develop the immune responses. Therefore, application of probiotic in the diet of immunized fish can prevent such negative effects on the growth condition as clearly seen in this study (a better growth condition in V+P group compared to others) as similar findings were seen by Soltani *et al*. (2017a, b) when vaccinated trout against *Yersinia ruckeri* were fed the same probiotic, an enhancement was seen in the growth factors and immunological responses compared to vaccinated fish fed normal feed.

Haematological parameters are important indicators of the general health condition of fish (Bandyopadhyay and Mohapatra, 2009; Kader *et al*., 2010). In this study, the WBC level in vaccinated trout, either with or without *L. plantarum* dietary supplement was significantly higher than other groups. Similar increases were also reported in Nile tilapia **(***Oreochromis niloticus*) and sturgeon (*Huso huso)* vaccinated against *A. hydrophila*, and trout vaccinated with polyvalent vaccine against three pathogenic species (Khosh-bavar-Rostami *et al*., 2007, Nikoskelainen *et al*., 2007; Silva *et al*., 2009). Also, a slight enhancement was seen in RBC and Hb and PCV in vaccinated fish fed with the probiotic. Such enchantment can be attributed to an increase in oxygen requirement due to the increase in metabolite rate in response to immunization (Fair *et al*., 1999; Pilorz *et al*., 2005). However, such differences were not significant compared to other trails. Yazici *et al*. (2015) reported that *L. plantarum* did not influence leukocytes counts of trout because their rearing condition was run in_sub-lethal water temperatures (25-26C°) that can negatively affect the immune status of the fish.

In conclusion, the present study explores the effects of *L. plantarum* on growth performance and haematological parameters of trout immunized with streptococcosis/lactococcosis vaccine. These results show that growth performance and some haematological indices i.e. WBC were improved in the vaccinated trout fed with the probiotic showing a synergistic effect by the probiotic and the vaccine. To our knowledge, this is the first study evaluating the beneficial efficacy of this probiotic as a dietary supplementation on growth and haematology of vaccinated trout against streptococcosis and lactoocosis.

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