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RESEARCH PAPER

Effects of *Lactobacillus plantarum* on Growth Performance, Proteolytic Enzymes Activity and Intestine Morphology in Rainbow Trout (*Oncorhynchus mykiss*)

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Abstract

In this study, effects of *Lactobacillus plantarum* on growth performance, proteolytic enzymes activity and intestine morphology of *Oncorhynchus mykiss* were studied. Juvenile rainbow trout (n=270) in 3 administration groups in 9 cement tanks were kept and fed with diets containing different doses (0, 0.3 and 0.5 g kg^{-1} feed) of the probiotic for 90 days. Results showed a positive effect on growth performance in both treatment groups compared to the control group. Acidic protease activity in the lower dose of administration was higher in comparison with the higher dose and control groups. Alkaline protease activity and trypsin activities showed the same pattern with the higher level in treatment groups compared to the control group. No significant difference was observed between probiotic groups and control in intestine morphology. The current study showed that *Lactobacillus plantarum* can improve growth performance and protease activity in juvenile rainbow trout.

Keywords: Rainbow trout, *Lactobacillus plantarum*, growth factor, enzyme activity, intestine morphology.

Introduction

Stressful rearing conditions render the cultured fish highly sensitive to different diseases. They are protected from infectious diseases by vaccination or chemotherapeutic treatment. However, due to extensive use of the chemotherapeutic agents, the occurrence of antimicrobial-resistance among pathogens and the associated environmental problems have been well documented (Ahilan, Shine, & Santhanam, 2004). Therefore, several alternative strategies including the use of probiotics have been proposed. Probiotics as feed supplements benefit the host by producing inhibitory compounds, competition for chemicals and adhesion sites, immune modulation and improving the microbial balance (Renuka, Venkateshwarlu, Ramachandra Naik, & Prashantakamara, 2013).

Lactic acid bacteria (LAB) are Gram-positive, non-spore forming and catalase negative rods that ferment various carbohydrates mainly to lactate and acetate. *Lactobacillus plantarum* (*L. plantarum*) is a member of one of the eight main genera of LAB that have been used as probiotics (Hagi, Tanaka, Iwamura, & Hoshino, 2004). This probiotic is known to produce antimicrobial substances like plantaricin that are active against certain pathogens (Cebeci & Gürakan, 2003). Previous studies demonstrated that *L. plantarum* can promote the growth, immunity and resistance against infectious agents in fish species (Son, Chang, Wu, Gu, & Chiu, 2009; Parthasarathy & Ravi, 2011; Giri, Sukumaran, & Oviya, 2013).

Improved growth performance in fish species can be attributable to different mechanisms (Heidarieh *et al.*, 2012). First, a reduction in pathogenic bacteria and an increase in positive bacteria in the gut of the treated fish can result in growth performance. Sometimes treated diet can influence nutrients especially protein digestibility by maintaining the function and structure of the small intestine, leading to an increased digestive capacity of the gut. Third, the addition of some diets can improve digestive enzymes. In this case, the high rate of secretion and release of digestive enzymes and the absorption of nutrients can guarantee a high energy budget that leads to increased growth rate (Nya & Austin, 2011).

In previous studies (Son *et al.*, 2009; Parthasarathy & Ravi, 2011; Giri *et al.*, 2013), increased growth rate following the administration of *L. plantarum* in fish species were noted. Until recently, simultaneous relationships between these additives and digestive enzyme activity as well as intestine morphology were not evaluated in the fish species. Therefore, present study was designed to evaluate the effects of *L. plantarum*on rainbow trout digestive enzymes in stomach and intestine besides intestinal histology.

Materials and Methods

Fish

Juvenile rainbow trout (28.8 ± 1.9 g, n = 270) were used in this study. They were kept at a fish farm in Firoozkooh, Iran, and distributed in 9 cement tanks (120 × 75 × 55 cm) filled with water to 45 cm depth. Each tank was continuously supplied with aerated free-flowing water with the flow rate set at 0.5 $L.s^{-1}$, water temperature 12 ± 2°C, and dissolved oxygen 8.5 ppm under natural light period (10L: 14D). Adaptation to these tanks was performed for seven days with commercial pelleted diet (Faradaneh, Iran). The analysis of this commercial diet is given in (Table 1). Fish were divided equally into three groups in triplicates with 30 fish in each tank. Probiotic *Lactobacillus plantarum* (KC426951) (National Center for Biotechnology Information, U.S.) was thoroughly mixed and added to the diet by spraying 20 ml fish oil kg^{-1} feed. A control diet was prepared by spraying 20 ml fish oil kg^{-1} only to the basal diet. Fish were fed diets containing different doses (0.3 and 0.5 g kg^{-1} feed) of probiotic in treatment groups (group1 and group 2) for 90 days. Fish were fed at the rate of 2.3% body weight six times daily at 07:30, 09:00, 11:00, 12:30, 14:00, and 16:00 h.

Growth Performance

Several growth factors namely final weight, weight gain, total length, condition factor (CF), visceral somatic index (VSI), specific growth rate (SGR) and survival rate were measured in this study (Nya & Austin, 2011).

Preparation of Crude Enzyme Extract

On day 90, ten fish samples were randomly selected from each tank and anaesthetized with clove powder (200 mg/l). Then, fish were dissected and specific regions of digestive tract namely stomach, pyloric caeca and intestine were extracted. Tissues from each region were washed with distilled water and weighed. The tissues homogenized in cold Tris–HCl 50 mM buffer pH 7.5, using a hand-held glass homogenizer. The homogenate was then centrifuged at 4°C at 10,000=g for 20 min. The supernatant containing the enzymes was stored at -70 °C before analysis.

Table 1. Proximate composition (% DWB) of the basal p

Proximate composition (%)	
Protein	41
Fat	16
Fiber	3.5
Ash	10
Moisture	11

Total Proteolytic Enzyme Activity

The total proteolytic enzyme activity was assessed from the stomach, intestine and pyloric caeca extract with the use of azocasein as the substrate according to Garcia-Carreno and Haard (1993). Briefly, 50 µL of enzyme homogenates was incubated with 0.5 mL of 0.5% azocasein in 50 mM Tris-HCl buffer, pH 7.5 at 25° C for 30 min before the reaction was ended with the addition of 0.5 mL of 20% trichloroacetic acid (TCA). Samples were centrifuged at 14600 g for 5 min and recorded at A440 for 3 min. For azocasein, one unit of proteolytic activity corresponds to the amount of enzymes releasing 1 µg azocasein per min. Under the assay condition, the extinction coefficient of azocasein (3648) was used for calculating proteolytic activity. For a control, TCA was added first to the sample before the addition of the substrate (Garcia-Carreno & Haard , 1993). Specific activity of the total protease activities was calculated as:

$$[Ab\ (supernatant) - Ab\ (control) / 30\ min \times mg\ protein] \times 100$$

Protein concentration in the samples was estimated according to Bradford (1976), using bovine serum albumin as standard.

Trypsin Activity

Trypsin activity was assayed from the intestine and pyloric caeca extract at pH 7.5 at 37°C, using benzoyl-argine-p-nitroanilide (BAPNA) as substrate according to Erlanger, Kokowsky, and Cohen (1961).

Enzyme homogenate (25µl) was mixed with Tris-HCl buffer (0.05 M, pH 7.5 containing NaCl (1 M) and CaCl2 (20 mM) before 1.25 mL BAPNA (1.0 mM) was added. After 10 min incubation at 37 °C, the reaction was stopped by adding 0.25 ml of 30% acetic acid and recorded at A410 for 3 min. Trypsin activity was calculated according to (Erlanger *et al*., 1961):

$$Ab\ (test/min) \times 1000 \times volume\ of\ reaction\ mixture / 8800 \times mg\ protein\ in\ the\ mixture$$

where 8800 is the molar extinction coefficient of p-nitroaniline.

Intestine Morphology

Samples of intestine (n=12) from each group were fixed in 10% buffered formalin for 48 h, dehydrated in ethanol solutions and xylene and then embedded in paraffin. A 5-micron subsample was then rehydrated in alcohols and stained for histological purposes with haematoxylin and eosin (H&E). Length and thickness of proximal intestinal villi, muscular layer and epithelium thickness were measured using a light microscope.

Statistical Analysis

Data were analyzed using one-way ANOVA, using the software program SPSS (Version 21.0) (SPSS) for windows. Differences between mean values were tested using Duncan's multiple range test (Duncan, 1955) and were considered statistically significant at the 5% probability level.

Results

Growth Performance

The effect of dietary *L. plantarum*on the growth performance of fish during a 90-day period of study is shown in Table 2. Results showed that parameters namely final weight, weight gain, total length, and SGR were higher in both treatment groups compared to the control group. CF and VSI in treatment groups were not significant in comparison with the control group. Moreover, survival rates were determined as 96, 90 and 80% for group 1, group 2 and control, respectively.

Total Protease Activities

Results of enzyme activities for the protease in the stomach, intestine and pyloric caeca are shown in Figure 1 and Figure 2. Higher protease activity in the stomach of group 1 was noted in comparison with group 2 and control group. On the other hand, protease activity in intestine and pyloric caeca of treatment groups showed the same pattern with higher levels compared to the control group.

Trypsin Activity

Trypsin activity in different groups is shown in Figure 3. Similar pattern to the alkaline protease in the intestine and pyloric caeca of treatment groups with higher level compared to the control group were noted.

Intestinal Morphology

The effect of dietary probiotic on the anterior intestine morphology was investigated (Table 3). Light microscopy of fish intestine showed normal appearance in all groups. No significant differences (P>0.05) was observed between the treatment group

Table 2. Growth parameters in juvenile rainbow trout administrated with *Lactobacillus plantarum*

	Control	Group 1	Group 2
Final weight (g)	115.8±0.89^{a}	160.9±1.3^{b}	165.59±1.4^{b}
Weight gain (g)	87±2.1^{a}	132.1±2.5^{b}	136.79±2.6^{b}
Total length (mm)	221±1.3^{a}	249±1.6^{b}	254±1.4^{b}
CF (%)	1.07±0.07^{a}	1.04±0.1^{a}	1.01±0.05^{a}
VSI (%)	12.4±0.3^{a}	11.8±0.7^{a}	12.1±0.6^{a}
SGR	1.5±0.1^{a}	1.91±0.2^{b}	1.94±0.2^{b}
Survival Rate (%)	80	96	90

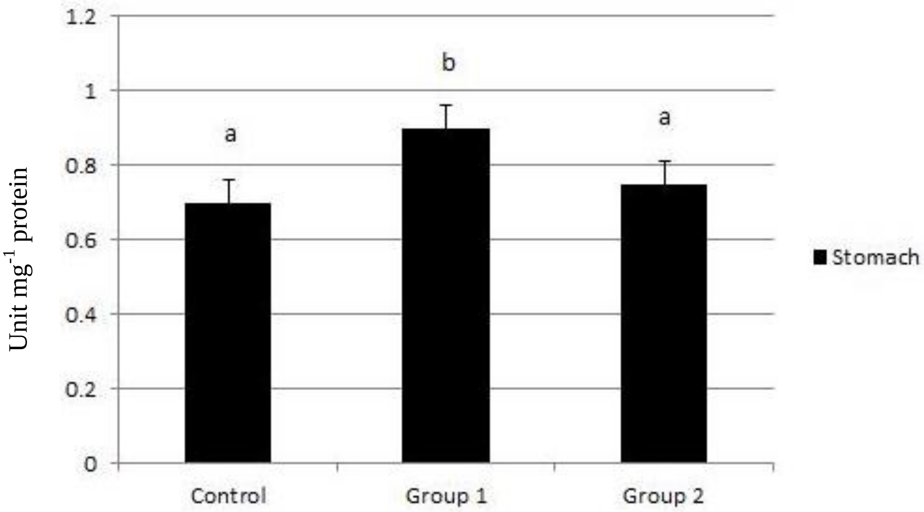

Figure 1. Protease activity of stomach in juvenile rainbow trout administrated with *Lactobacillus plantarum*.

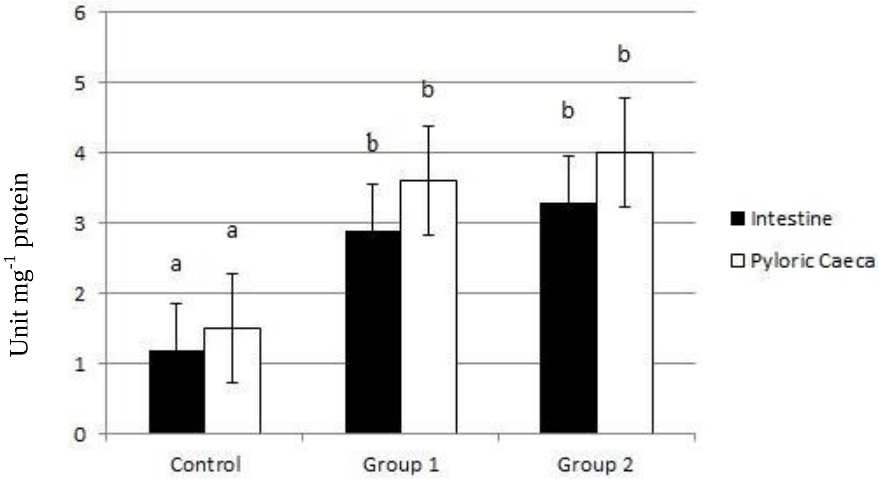

Figure 2. Protease activity of intestine and pyloric caeca in juvenile rainbow trout administrated with *Lactobacillus plantarum*.

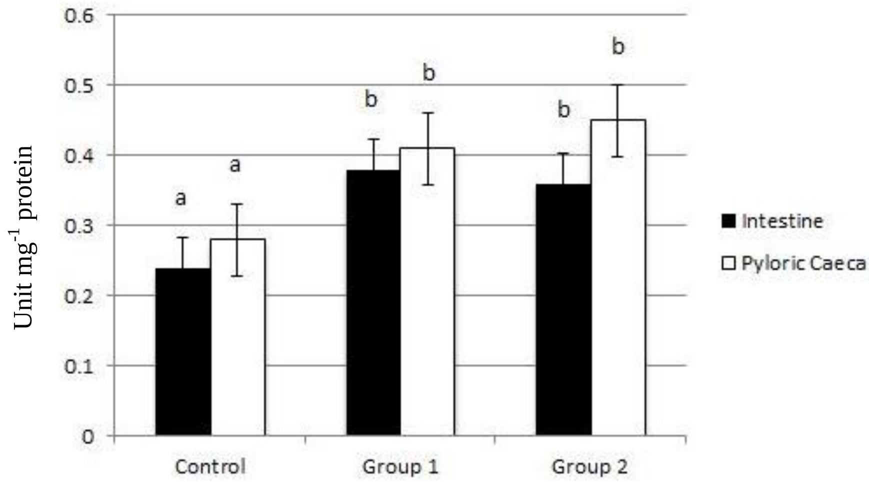

Figure 3. Trypsin activity of intestine and pyloric caeca in juvenile rainbow trout administrated with *Lactobacillus plantarum*.

Table 3. Intestinal morphological parameters in juvenile rainbow trout administrated with *Lactobacillus plantanum*

	Control	Group 1	Group 2
Villi Length (µm)	1324.15±87.3	1356.49±80.2	1264.58±82.6
Villi Thickness (µm)	126.58±20.1	121.17±18.7	119.16±19.7
Muscular Layer Thickness (µm)	80.97±12.3	73.79±11.3	78.02±17.6
Epithelium Layer Thickness (µm)	137.58±22.4	131.33±25.1	143.27±23.1

and the control in terms of muscular layer thickness, epithelium layer thickness, villi length and thickness.

Discussion

Probiotics, which are micro-organisms or their products with health benefits for the host, have been used in aquaculture as a means of growth enhancement and diseases control, to supplement or even in some cases to replace the use of antimicrobial compounds (Iman *et al*., 2013). This study was conducted to investigate the effects of *L.plantarum* as probiotic on growth performance, some proteolytic activities and intestinal morphology of *O.mykiss*. Results of this study showed that growth performance of different doses of probiotic *L.plantarum* used over 90-days caused significant improvements in rainbow trout final weight, weight gain, total length, SGR and survival rate compared with the control group ($P<0.05$). These results agree with various reported researches. Similarly, dietary *L. plantarum* administration at 10^8 cfu kg^{-1} promoted growth in grouper (*Epinephelus coioides*) (Son *et al.*, 2009). In the study by Giri *et al.* (2013) *L.palntarum* was added to *Labeo rohita* diet. Daily weight growth (WG) and feed conversion ratio (FCR) showed a significant increase. The improvement of growth performance for fish fed a diet supplemented with *L.plantarum* could be due to different mechanisms. Intestinal morphology can affect the physiology and metabolism of nutrient absorption which result in different growth performance. In the current study, the intestine morphology was analyzed with a light microscope, evaluating influence of the probiotic *L.plantarum* on muscularis layer thickness, epithelium layer thickness and villi length and thickness. Those parameters were not affected significantly by the probiotic. Despite the importance of the intestine as a digestive, absorptive and defensive organ, only a few studies have investigated the effect of probiotics on intestinal morphology which showed controversial results. For example, the dietary incorporation of other probiotic bacteria (*B.subtilis*, *B.licheniformis*, and *Enterococcus faecium*) did not affect the intestinal morphology (Merrifield, Harper, Dimitroglou, Ringo, & Davies, 2010). Conversely, probiotic *Lactobacillus rhamnosus* administration produced an increase in the villus height of Nile tilapia (Pararat *et al*., 2011). Meanwhile, rainbow trout fed a diet supplemented with *Pediococcus acidilactici* showed increased villi length but not density (Merrifiel *et al*., 2010). In the study of aquatic animals, intestinal villus length and mucosal folds are considered as a sign of absorptive ability. The larger size of villi can result in a greater absorptive intestinal surface, enabling improvement in nutrient absorption, which might explain the moderate numerical improvement in growth performance in fish species fed the probiotic supplemented diet (Gisbert, Castillo, Skalli, Andree, & Badiola, 2014). Controversial effects about nutritional components on intestinal morphology may be due to factors such as, differences in fish species, dietary inclusion levels, age and health status of fish used.

Digestive enzymes are among the most important factors that influence the efficiency of feed utilization in fish and hence characterization of these enzymes provides important information regarding the digestive capacity of fish to hydrolyze carbohydrates, proteins and lipids in feed ingredients (Lemieux, Blier, & Dutil, 1999). The present results characterized the effect of using a different level of *L.plantarum* probiotic in rainbow trout on total protease and trypsin activities and showed a significant difference in treatment groups especially lower dose of administration compared to the control group. As previous studies suggested (Suzer *et al*., 2008), probiotics can influence digestive processes by enhancing the population of beneficial microorganisms, improving the intestinal microbial balance and microbial enzyme activity, consequently improving the digestibility and absorption of food and feed utilization, which was demonstrated in this study by better growth performance and survival rate in treated groups. Similar studies have shown probiotics significantly affect in the digestion process (Munilla-Moran, Stark, & Barbour, 1990) by increasing the total protease activity of the gut (Ziaei-Nejad *et al*., 2006) and stimulating the production of endogenous enzymes (Ochoa-Salano & Olmos-Soto, 2006; Wang, 2007) which in turn can increase the food digestibility. Additionally, the exogenous enzymes have a broader pH range than endogenous enzymes that lengthens the digestion time and may permit better hydrolysis of substrates.

Other mechanisms related to higher growth performance following the administration of probiotics were also found. In previous study (Al-Dohail, Hashim, & Aliya-paiko, 2009), lower levels of cortisol in fish fed the probiotic diet were shown. Therefore, lower stressor levels in these fish could result in better growth performance and nutrient efficiency.

In conclusion, present study showed the positive effects of *L.plantarum* supplement (KC426951) on growth performance and proteolytic enzyme activities in juvenile rainbow trout whereas intestine structure did not show significant differences in treated fish. Further research needs to be conducted to evaluate the exact effects of probiotic on different mechanisms of actions simultaneously to understand the efficient mechanisms behind the higher growth performance in treated fish.

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