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LONG-TERM EFFECTS OF PROBIOTICS ON HEALTH BIOCHEMICAL INDICES OF SERUM AND MUCUS IN RAINBOW TROUT (*Oncorhynchus mykiss*)

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Abstract

The object of this study was to evaluate the long-term effects of *Lactobacillus fermentum, Lactobacillus buchneri* and *Saccharomyces cerevisiae*, on serum and mucus biochemical indices as health indicator of rainbow trout (*Oncorhynchus mykiss*) at growing phase. A total of 160 fish with average weight of 250±50 g was divided into 8 groups and fed by diet supplemented with 10^7 CFU g^{-1} of each probiotic for 60 days in singular or combined feeding trail. The results showed that long-term feeding of probiotic supplemented diet increased serum alanine aminotransferase while reduced both serum and mucus alkaline phosphatase level. The probiotic supplements had no significant effects serum aspartate aminotransferase and mucus total protein by days 30 or 60 of experiment. In conclusion the results of this experiment showed that long-term feeding on probiotic supplemented diets may have adverse effects on health of rainbow trout and accordingly, it is not recommended to feed fish for more than 30 days on probiotic supplemented diets.

Keywords: Probiotic, rainbow trout, health biochemical indices; feeding time

Introduction

Today, aquaculture is the main way of increasing the production of aquatic animals. In recent decades, the aquaculture industry has grown with a higher annual growth rate rather than other sections in animal protein production (FAO, 2016). Of course, along with this significant growth, there has always been problems accompanied with aquaculture such as poor water quality and the prevalence of viral, bacterial, and fungal infections. The outbreak of diseases is a major problem in aquaculture, which has affected its development in many countries around the world (Awad & Awaad, 2017; Harikrishnan et al., 2009). To prevent, treat or control infectious diseases in fish, vaccines or chemical drugs, including antibiotics, are commonly used. However, the widespread use of antibiotics causes antibiotic resistance to pathogenic microorganisms and environmental hazards along with bacterial resistance in these animals (Acar et al., 2015; Awad & Awaad, 2017; Van Hai, 2015; Yilmaz, 2019).

Various studies confirmed that probiotics are appropriate alternatives for antibiotics to remove the cycle of antibiotic resistance (Wang et al., 2017). Probiotics have been used in human and animal nutrition and recently in aquaculture. The use of probiotics is, in fact, a new aquaculture technology that is environment friendly. Probiotics are defined as microbial cells or compounds that have beneficial effects on their host's health (Balcázar et al., 2006). These organisms are added to commercial foods as supplements and improve the balance of the gastrointestinal flora, and is used to prevent the spread of diseases, increase the efficiency of the conversion factor, stimulate growth, balance and strengthen the immune system and resistance to stress (Saad et al., 2013). Microorganisms as probiotics used in aquaculture include yeasts, bacteria and algae (Argyri et al., 2013; Wang et al., 2008).

Previous studies have clearly shown that the use of food supplements such as probiotics and prebiotics can improve the immune system through various mechanisms, including increasing the level of activity of immune parameters, which in turn increases resistance to diseases and environmental stressors, which subsequently results in increasing the survival of fish. So far, many studies have been done on the effects of probiotics on immune system in fish and the positive effects have been confirmed (Das et al., 2013a). However, most studies have focused on short term use of probiotics on early life stages while several widespread diseases occur during fattening period in aquaculture. Also, there are limited information on the combined use of probiotics on the health indicator of fish. So, the main object of this study was to evaluate the long-term effects of *L. fermentum, L. buchneri* and *S. cerevisiae*, on serum and mucus biochemical indices as health indicator of rainbow trout (*O. mykiss*) at growing phase.

Material and Method

Experimental Fish

For this study, a total of 160 rainbow trout (*O. mykiss*) with an average weight of 250±50 g was provided from a private commercial farm in Sepidan, Fars Province (Iran) and transferred to the laboratory and kept in Semi-recirculating Aquaculture System (RAS) occupied with aeration and circulating equipment. After 15 days of acclimatizing to pre-described condition, the fish were randomly divided into 8 groups in duplicate each of 20 individuals. Water temperature and dissolved oxygen levels were recorded daily and kept in at optimum range for rainbow trout. Water temperatures ranged from 14.3 to 16.5 ºC and dissolved oxygen was kept above 6 mg l^{-1}.

Preparation of Experimental Feed

Commercial rainbow trout (*O. mykiss*) feed was provided from Beyza Feed Mill, Shiraz, Iran. The ingredient percent of diets were as 42% crude protein, 15.5% crude fat and 4400 kcal/kg energy. *L. fermentum* (ATCC 14931) and *L. buchneri* (PRM 205) provided by Roshdgostar Mehregan company in Shiraz, Iran and *S. cerevisiae* at 10^{10} CFU g^{-1} were purchased from ARDEYPHARM, Germany. To obtain a pure colony, the bacteria were first cultured in MRS Agar (De Mann, Rogosa, Sharpe), Merck, Germany, and then a pure colony was transferred to MRS Broth (Merck, Germany) and incubated for 24 hours at 37 °C. The bacterium was grown to 10^8 CFU / mL (MRS Broth), and finally, 100 cc of MRS Broth was sprayed on one kilogram of basal diet to prepare the desired concentration (10^7 CFU per gram of food), then coated with bovine gelatin solution for preventing water leaching. In order to make the experimental diet containing bakery yeast (*S. cerevisiae*), the yeast at concentration 10^{10} CFU g^{-1} dissolved in 100 ml saline solution and sprayed on basal diet to obtain final concentration of 10^7 CFU per gram of feed. The diet containing yeast was also covered with bovine gelatin solution to decrease the leaching. Fish were fed twice daily according to the water temperature and biomass of each tank following manufacturer recommendation. The details of experimental groups are presented in Table 1.

Table 1. Experimental diet used in research. All diets coated with bovine gelatin to prevent leaching as described in text.

Treatment groups	Description
C	Control group (without any prebiotic)
F	*L. fermentum* (10^7 CFU per gram of diet)
B	*L. buchneri* (10^7 CFU per gram of diet)
F+B	*L. fermentum* + *L. buchneri* (1:1 W/W)
S	*S. cerevisiae* (10^7 CFU per gram of diet)
S+F	*S. cerevisiae* + *L. fermentum* (1:1 W/W)
S+B	*S. cerevisiae* + *L. buchneri* (1:1 W/W)
F+B+S	*L. fermentum* + *L. buchneri* + *S. cerevisiae* (1:1:1 W/W)

Sampling

Fish were bled by days 30 and 60 after anesthetization by clove powder at dose 150 mg l^{-1}. At each sampling time six fish were randomly taken from each treatment. Blood samples were centrifuged at 10000 rpm for 15 min after overnight at 4 ºC and the sera were kept at -20 ºC for further analysis.

Mucus samples were also collected on days 30 and 60 as described previously (Subramanian et al., 2007) with some modifications. Briefly, three fish were randomly selected and anesthetized with clove powder (150 mg L^{-1}). In order to minimize the bacteria attachment to the body and to eliminate other contaminants, the fish were washed with tab clean water and immediately were put in bags containing 9 mL of PBS. After 2 min., the fish were transferred to a proper oxygenated

tank. The mucus samples were centrifuged (K241R, UK) at 1500 rpm for 10 minutes at 4 °C and the supernatants were separated and kept in -20 °C until use.

Measurement of Biochemical Parameters

Total protein (TPR) of mucus samples was measured by Biuret method using commercial kits (Pars Azmun, Iran) following the manufacturer's protocol. Aspartate aminotransferase (SGPT), alanine aminotransferase (SGOT) and alkaline phosphatase (Atamanalp & Yanik, 2003) were also measured using commercial kits (Pars Azmun, Iran) following the manufacturer's protocol. The absorbance of samples was read by using an auto-analyzer (Alpha Classic-AT ++, SANJESH CO).

Statistical Analyzes

Data were analyzed as repeated measurement to elucidate the main effects of treatment, time and their interactions using GLIMIX procedure in SAS 9.1.4 software. The normality of data was checked by Shapiro-Wilk test. The covariance structure was compound symmetric. In order to distinguish the differences among treatments over time, the mean squares of the treatments were compared by Tukey post-hoc test at the significant level of $P < 0.05$. Data were presented as means ± Standard Error of Mean (SEM).

Results

The main effects of time and treatments are presented in Table 1 and 2 respectively. Also, the interaction effects of treatment×time are presented in Figure 1 and 2.

Table 2. Main effects of sampling time on the biochemical parameters of serum and mucus of rainbow trout (*O. mykiss*) fed on diets supplemented with different treatments of probiotics *L. fermentum, L. buchneri* and *S. cerevisiae*.

Index (g dL^{-1})	30 Days	60 Days	*p-value*
Serum SGOT	2.2 ± 0.05 [b]	2.3 ± 0.05 [a]	0.04
Serum SGPT	1.1 ±0.12 [a]	1.5 ± 0.12 [a]	0.07
Serum ALP	2.9 ± 0.02 [a]	2.8± 0.02 [b]	0.04
Mucus TPR	0.02 ± 0.01 [a]	0.01 ± 0.01 [a]	0.12
Mucus ALP	1.1 ± 0.03 [a]	0.08 ± 0.03[b]	0.01

Data are reported as mean ± S. E. M. Control; F: *L. fermentum*; B: *L. buchneri*; S: *S. cerevisiae.* ALP: Alkaline phosphatase; TPR: Total protein; SGPT: Aspartate aminotransferase; SGOT: Alanine aminotransferase.

Liver Enzymes

SGOT

Results in Table 2 demonstrated that the serum level of SGOT significantly increased on day 60 of the experiment in all probiotic treatments compared to day 30 ($P < 0.05$). Also, after 60 days of feeding, a significant difference was observed among all treatments ($P < 0.05$). The highest level of SGOT was observed in group F (2.4 ± 0.09 g dL^{-1}) and the lowest level (2.1 ± 0.09 g dL^{-1}) in F+B+S treatment (Table 3). However, the results of the interaction of treatment×time analysis (Figure 1A) showed that there was no significant statistical difference among the treatments on the 30^{th} and 60^{th} day of the experiment ($P \geq 0.05$).

Table 3. Main effects of treatments on the biochemical parameters of serum and mucus of rainbow trout (*O. mykiss*) fed on diets supplemented with probiotics *L. fermentum, L. buchneri* and *S. cerevisiae*.

Treatments	Mucus ALP (g dL^{-1})	Mucus TPR (g dL^{-1})	Serum ALP (g dL^{-1})	Serum SGPT (g dL^{-1})	Serum SGOT (g dL^{-1})
C	0.95 ± 0.05^{ab}	0.02 ± 0.006^{a}	$2.8 \pm 0.03^{/a}$	1.2 ± 0.26^{a}	2.2 ± 0.09^{ab}
F	1.07 ± 0.05^{ab}	0.02 ± 0.006^{a}	2.7 ± 0.03^{ab}	1.3 ± 0.26^{a}	2.4 ± 0.09^{a}
B	0.92 ± 0.05^{ab}	0.01 ± 0.006^{a}	2.8 ± 0.03^{a}	1.3 ± 0.26^{a}	2.2 ± 0.09^{ab}
F+b	0.93 ± 0.05^{ab}	0.02 ± 0.006^{a}	2.7 ± 0.03^{ab}	1.1 ± 0.26^{a}	2.3 ± 0.09^{ab}
S	1.03 ± 0.05^{ab}	0.02 ± 0.006^{a}	2.8 ± 0.03^{a}	1.1 ± 0.26^{a}	2.3 ± 0.09^{ab}
F+s	1.00 ± 0.05^{ab}	0.04 ± 0.006^{a}	2.8 ± 0.03^{a}	1.4 ± 0.26^{a}	2.3 ± 0.09^{ab}
B+s	1.08 ± 0.05^{a}	0.01 ± 0.006^{a}	2.8 ± 0.03^{a}	2.0 ± 0.26^{a}	2.2 ± 0.09^{ab}
F+b+s	0.79 ± 0.05^{b}	0.01 ± 0.006^{a}	2.6 ± 0.03^{b}	1.1 ± 0.26^{a}	2.1 ± 0.09^{b}
p- value	0.01	0.07	0.01	0.34	0.01

Data are reported as Ls-mean ± S. E. M. C: Control; F: *L. fermentum*; B: *L. buchneri*; S: *S. cerevisiae.* ALP: Alkaline phosphatase; TPR: Total protein; SGPT: Aspartate aminotransferase; SGOT: Alanine aminotransferase.

SGPT

The results showed that the main effect of time was not significant for SGPT and accordingly, there was no statistically significant difference in serum SGPT by days 30 or 60 ($P \geq 0.05$) (Table 2). Also, the main effects of treatments were not significant, so no significant statistical difference was observed between the probiotic treatments and the control group ($P \geq 0.05$) (Table 3). Also, the interaction of time×treatment was not significant. Accordingly, there was no significant difference among the probiotic treatments and the control group in case of SGOT on different sampling days ($P \geq 0.05$) (Figure 1B).

ALP

Based on the results of this study, the amount of serum ALP among all probiotic-containing treatments on days 30 was significantly higher than those at 60th day of the experiment in rainbow trout (*O. mykiss*) ($P \leq 0.05$) (Table 2). Also, the results showed that there was a significant statistical difference among the treatments after 60 days of feeding ($P \leq 0.05$). The lowest level (2.6 ± 0.03V) was observed in the F+B+S treatment (Table 3). The interaction of time×treatmnet showed that there was also a significant difference in the amount of this parameter among the different treatments on the 30th and 60th day of feeding ($P \leq 0.05$). The lowest level (2.71V) belonged to the F+B+S treatment on the 30th day and the F treatment (2.74) on the 60th day (Figure 1C).

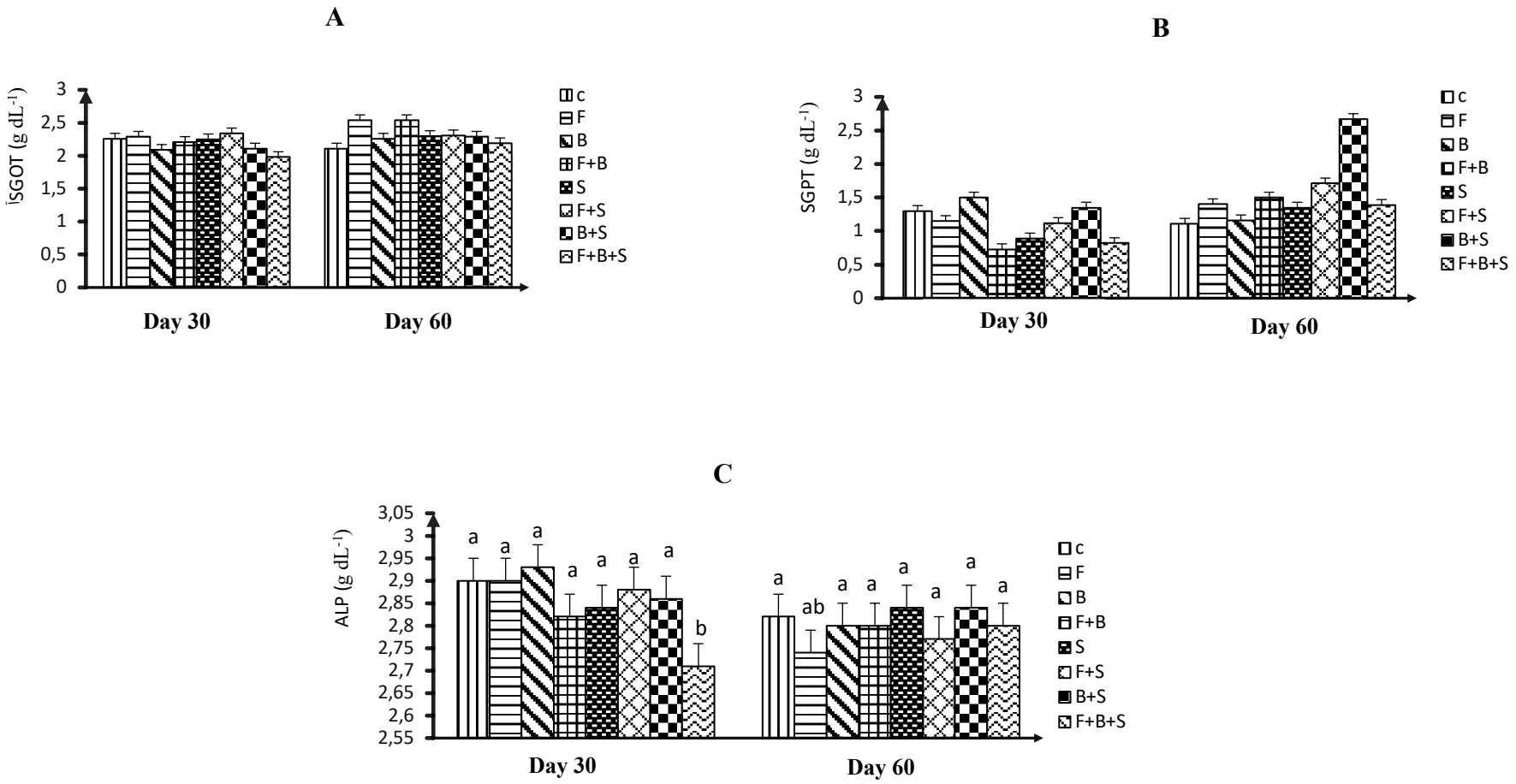

Figure 1. Interaction effects of treatment×time on the serum SGOT (A), SGPT (B) and ALP (C) of rainbow trout (*O. mykiss*) fed on diet supplemented with probiotics *L. fermentum, L. buchneri* and *S. cerevisiae* at different sampling time. Data are reported as Ls-mean± S. E. M. Bars with different letters are significantly different at P≤0.05. C: Control; F: *L. fermentum*; B: *L. buchneri*; S: *S. cerevisiae.* Data for ALP and SGOT were log transferred and data for SGOT was rooted for normalization.

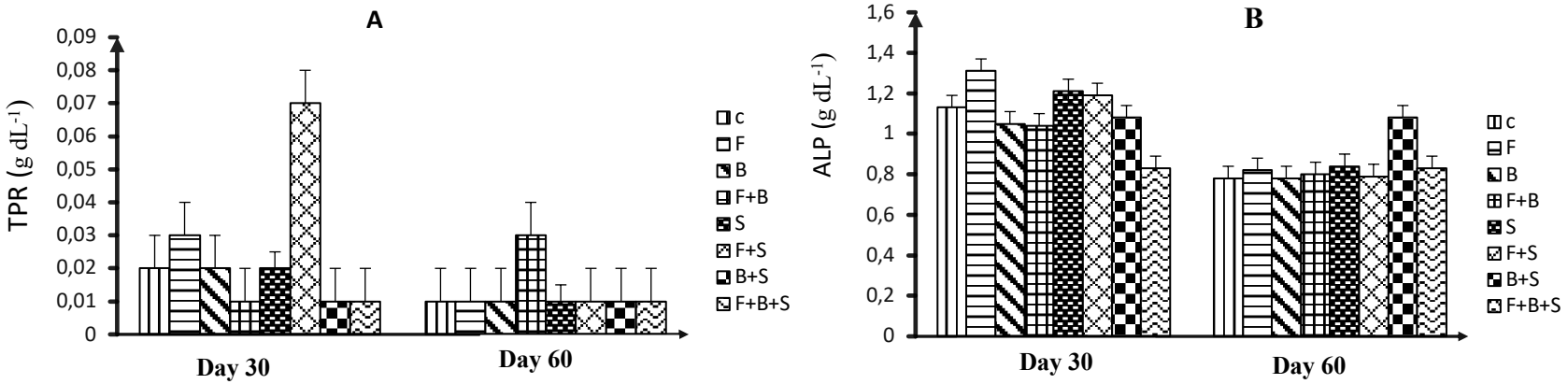

Figure 2. Interaction effects of treatment×time on the mucus TPR (A) and ALP (B) of rainbow trout (*O. mykiss*) fed on diet supplemented with probiotics *L. fermentum, L. buchneri* and *S. cerevisiae* at different sampling time. Data are reported as Ls-mean±SEM. No significant differences were observed among means. C: Control; F: *L. fermentum*; B: *L. buchneri*; S: *S. cerevisiae.* Data for ALP and SGOT were log transferred and data for SGOT was rooted for normalization.

Mucus Biochemical Parameters

ALP

Based on the results of this study, there was a statistically significant difference in mucus ALP level in rainbow trout (*O. mykiss*) between days 30 and 60 (0.05). The level of this parameter reduced on the 60th day of feeding compared to the 30th day (Table 2). Also, there was a significant difference among the treatments after 60 days of feeding (P≤0.05). Accordingly, the highest and the lowest level of mucus ALP were observed in S+B (1.08 ± 0.05V) and F+B+S groups (0.79 ± 0.05V), respectively (Table 3). However, the results of treatment×time analysis showed that there was no significant difference in the amount of this parameter among different treatments (P≥0.05) (Figure 2A).

TPR

The results of this study showed that the main effect of time was not significant in case of TPR (P≥0.05) (Table 2). There was also no significant difference among treatments during the 60 days of feeding (P≥0.05) (Table 3). The results of treatment×time analysis also showed that although the level of TPR in group F+S was higher than those in other treatments, there was no significant statistical difference in case of mucus TPR among the treatments at the 30th and 60th day of feeding (P≥0.05), (Figure 2B).

Discussion

Biochemical characteristics play an important role in the physiological health of fish, which provides useful information on the health status of the fish (Ahmadi et al., 2014). Because of the fact that all the substances are metabolized in the liver after entering the body, and their metabolites are transported to the kidneys, hence these two organs have the greatest effect on the absorption of the substances (Lemaire et al., 1991).

The increased activity of SGOT and SGPT enzymes in plasma indicates tissue damage, especially in the liver and heart, however these enzymes are not specific to the liver and they are usually made in liver, kidney, heart cells, skeletal muscle, kidney, pancreas, spleen, red blood cells and gills (Agrahari & Gopal, 2009). The evaluation of liver enzymes is commonly used to measure liver damage in different species of fish. Increasing the level of these enzymes in blood occurs when the liver cells are damaged.

The ALP enzyme is commonly found in most fish tissues and is usually produced by the gallbladder cells (Tamás et al., 2002) ALP is a hydrolysable enzyme responsible for removing phosphate groups from a variety of molecules, including nucleotides and proteins. As its name suggests, it works best in alkaline environments. The enzyme also has a multifunctional role, such as immunity, and has been showed to reduce after contamination with pathogens and it could be accompanied with suppressing the immune system (Waagbø et al., 1988). In this study, there was no significant difference in the protein level of mucus in the probiotic-receiving treatments and the control group on the 30th and 60th day of the experiment. There was also no difference between the use of probiotics as individual or in combination.

Based on the results of this present study, the interaction of treatment×time was not significant for both SGOT and SGPT but the amount of SGPT increased over times and in some probiotic receiving treatments. The amount of SGOT increased significantly on the 60th day compared to the 30th day, although this was not the case for SGPT. In contrast, the level of alkaline phosphate significantly decreased on 60th day of sampling in comparison to day 30. In line with the results of our study, Tonekabon (2013) reported that adding bacterial probiotics *B. Subtilis* with a dose

of 10^7 CFU g^{-1} to rainbow trout (*O. mykiss*) diet for 60 days has no effects on SGOT and SGPT, but it results in a significant difference in ALP level in the probiotic treatments compared to the control group. It has also been reported that feeding Nile tilapia (*Oreochromis niloticus*) with *Pediococcus acidilactici* bacteria with a dose of 10^6 CFU g^{-1} for 6 weeks has no significant effect on the level of SGOT, SGPT and ALP (Standen *et al*., 2013). Also 7 weeks of feeding *Huso huso* with oligofructose does not affect the level of SGOT, SGPT and ALP (Hoseinifar et al., 2011). However, (El-Rhman et al., 2009) reported that after 90 days of feeding bacterial probiotic *M. luteus* and *Pseudomonas* at a dose of 10^7 CFU g^{-1} individually or in combination, caused a significant difference in the level of SGOT and SGPT in Nile tilapia among probiotic treatments and the control group and the highest level was observed in probiotic-containing groups, but the difference between individual or combined use of probiotics was not reported.

Different results have been reported on the effects of probiotics on fish, which can be due to the differences in biology, the physiological status of different species, the composition of nutritional formula, or other environmental factors (Al-Dohail et al., 2009; Kumar et al., 2008).

It has been reported that the increase in the level of SGOT and SGPT in the serum of fish fed with probiotics might indicate the side effects of probiotic supplements on hepatocytes or other cells in fish, and cause pharmacological toxicity in fish cells (Lemaire et al., 1991). Therefore, considering using probiotics in this study for 60 days that increased the amount of SGOT and SGPT and reduced the amount of ALP in rainbow trout (*O. mykiss*), it is possible that the long-term use of these probiotics might increase the levels of these enzymes through producing undesirable substances, and so it may have a negative effect on fish. Regarding the improvement of liver enzymes after 30 days and ineffective or negative effects on the assays after 60 days of feeding, it can be suggested that probiotics used in this study can affect the liver enzymes in the short term, but may have negative consequences in long-term use. In previous studies, it has been suggested that probiotics should be used at least for two weeks, so that microorganisms can be colonized in the intestine (Das et al., 2013b).

Conclusion

In conclusion, the results of this research recommended to not use of probiotics for more than 30 days in rainbow trout (*O. mykiss*) at growing phase due to its possible negative effects on health indicators.

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Ethical approval

The experiment was performed under the approval of the State Committee on Animal Ethics, Shiraz University, Shiraz, Iran. Also, the recommendations of the European Council Directive (86/609/EC) of November 24, 1986, regarding the standards in the protection of animals were used for experimental purposes.

Informed consent

Not available.

Data availability statement

The authors declare that data are available from authors upon reasonable request.

Conflicts of interest
There is no conflict of interests for publishing of this study.

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Contribution of authors
All authors in this study have equally contributed in terms of conceptualization, data curation, formal analysis, writing original draft, funding acquisition, investigation, methodology, resources, validation, and visualization, and finalizing paper.

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