Probiotic supplements as an alternative medicine; investigation the Effect of *Lactobacillus casei* on liver function of Koi Fish (*Cyprinus rubrofuscus* L.) in exposure to pathogen as an animal model

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Abstract

Some infectious diseases, such as Salmonella in ornamental fish, are important because of their potential for human transmission and antibiotic resistance. In this study, 250 Koi fish with an average length of 10±3 cm and a weight of 20±1 gr were randomly divided into four groups with two repetitions. The test was performed for 24 days with diet and 72 hours' exposure to Salmonella Typhimurium. Fish are classified into four groups T1; receiving *lactobacillus casei* (1.5 × 108 CFU / ml) probiotic and not exposed to the pathogen, T2; getting probiotics and exposed to the pathogen, T3; no probiotics received but exposed to the pathogen, and control group (C). In the present investigation, to evaluate the damage caused by Salmonella typhimurium, Alkaline phosphatase (ALP), Alanine aminotransferase (ALT), and Aspartate transaminase (AST) were tested on days 0, 24 and 27 of the experiment. Based on the results, the effect of *Lactobacillus casei* probiotic in comparison with the control group on the improvement of liver function in this species of fish was investigated in such a way that the presence of probiotics alone may cause liver function in fish under normal or optimized conditions. However, the group that was not exposed to any probiotic agents showed a clear increase in the level of ALP, AST and ALT in the liver, which indicates the destruction of liver cells ($p<0.05$). However, in the group exposed to the pathogen along with the probiotic agent, the pathogen first increased the amount of some enzymes, but finally, with the action of probiotic factors, a decrease in enzyme levels in fish can be seen ($p<0.05$). In the third group, which did not use any probiotics, to increase the level of liver function enzymes in fish is observed significantly ($p<0.05$). In the study of three main indicators of liver diagnosis, it was found that one of the proposed ways to have healthy fish both from the point of view of aquaculture and as an animal model; using probiotics such as *Lactobacillus casei* can significantly improve liver and therefore metabolic function.

Keywords: Lactobacillus casei, Salmonella typhimurium, Koi fish, Cyprinus Rubrofuscu, ALT, AST, ALP

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Introduction

Aquaculture is an important economical priority in many countries. Stressful conditions and deterioration in the environment may result in serious economic losses. This may occur when large-scale productions perform, where aquatic animals are exposed to pathogens (Hasan and Banerjee, 2020).

Salmonella is a gram-negative bacterium that has been identified in aquatic life for many years (Liu *et al*., 2018).

Because of the potential of infection transmission to humankind and antibiotic resistance problem considering to infected ornamental fish, is important (Zhang *et al*., 2019).

Use antibiotic is one of the routine ways against infectious factors.

However, the utilization of antibiotics as preventive agents against infection has been questioned. There is extensive documentation of the evolution of antimicrobial resistance among pathogenic bacteria (Roope *et al*., 2019).

A very strong selection pressure towards resistance among bacteria to these amounts of antibiotics which have exerted happens to adapt them to this situation, mainly by a horizontal and promiscuous flow of resistance genes (Peterson and Kaur, 2018).

Today utilization of probiotics the beneficial bacteria, which control pathogens through a variety of mechanisms, is an important approach increasingly viewed as an alternative method against antibiotic treatment. The use of probiotics in human and animal nutrition is presented in documents (Yim *et al*., 2019)

Use of probiotics in addition to being used for increasing survival rates in aquacultural and ornamental fish, including Koi fish, probiotics can be used as an alternative medicine against antibiotics (Hasan and Banerjee, 2020).

Materials and methods

The Koi fish were supplied (n=120) with an approximate size of 10±3 cm in average weight of 20 grams from the Ornamental fish aquaculture and breeding center located in Shahriar-Tehran. To make the animal compatible with surrounding related factors they were transferred to Razef Research Complex located in Islamic Azad University, Science and Research branch, Shahriar-Iran.

Salmonella Typhimurium was supplied in lyophilized form, from the Faculty of Veterinary Medicine of Tehran University of Veterinary Medical Sciences.

Lactobacillus casei was supplied in lyophilized form, from the Microbial Bank of Iran Biological Resources Center. To confirm the bacterial strain and characteristics approval microscopic examination and biochemical tests were performed.

Lactobacillus casei cultured in 24 hours at 37°C in microbial MRS medium. To dilute the desired concentrations and prepare a 0.5 McFarlandturbidity ($1.5x10^8$ CFU /ml), using the optimal density method, 0.9% normal saline

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was used (Mahdavi and Isazadeh, 2019).

After a period of adaptation with light: dark conditions in 14:10 hours in optimum temperature, Koi fish were randomly categorized into 4 groups with two experiments replicate in 12 tanks.

The classification was as follow, T1; feeding with probiotic diet and no exposure to *S. Typhimurium*, T2; the Probiotic supplement diet with exposure to *S. Typhimurium,* T3; feeding with commercial nutrients and they were exposed to *S. Typhimurium* and the control group which was fed with commercial nutrients with no exposure to *S. Typhimurium*.

Nutritional diet was arranged based on 5% of the bodyweight of fish and fed twice a day, the diet contains the basic diet with probiotics in combination and also the basic diet with equal amounts of PBS, for 24 days twice a day at 9 a.m. and 4 p.m. (Tachibana *et al.*, 2020). At the end of the feeding period, T2 and T3 groups were exposed to *S. Typhimurium* for 72 hours (Wanka *et al*., 2018).

Sampling was done to evaluate liver serum enzymes in the first days, after the feeding period and after the end of exposure, from all replications by blood sampling from the tail of fish. The samples prepared for biochemical tests were immediately transferred to the laboratory in the icebox and the test was performed using the commercial kit of Pars Azmoon Company.

Results

Based on the study of liver enzyme levels, evidence suggests that in the T1, who were fed only probiotic agents, over time, from days 0 to 24 and 27, liver function status improved slightly, which in Figures 1, 2 and 3 can be seen. Although this difference was not statistically significant in the study of Alkaline phosphatase, the two enzymes Aspartate aminotransferase and Alanine aminotransferase in comparison between the groups of days 24 and 27 was meaning full ($p<0.05$). The second group called T2, which was exposed to pathogens in addition to the probiotic agent showed an increase in the level of alkaline phosphatase enzyme on the twenty-fourth day compared to the first day, which indicated the approximate action of the pathogen on liver activity, but on the twenty-seventh day, the decrease was seen to indicate the resistance of the probiotic agent to the pathogen ($p<0.05$)(Fig. 1).

The behavior of the third group (T3) fed with a normal diet and pathogens showed that the pathogen gradually harmed liver activity and causes more destruction of liver cells ($p<0.05$), while the normal and control groups in terms of alkaline phosphatase enzyme levels. They lived in almost equal conditions and without statistical differences ($p<0.05$).

Study of serum Alanine aminotransferase levels in probiotic-exposed Koi fish in the first group, T1, revealed that serum Alanine transaminase levels decreased sharply

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during exposure to probiotic, and this significant decrease with the first-day level indicated that probiotic could be a successful approach in improving liver function ($p<0.05$), while in the T2, which fed with the probiotic and also been exposed to pathogens, initially there was a slight increase in blood levels of mentioned liver enzyme, without any statistical significance ($p<0.05$). But on the 27th day, a decrease in the level of Alanine aminotransferase was observed, which lacks statistical significance ($p<0.05$). In the third group, the use of normal diet with pathogens results revealed the rate of liver cell destruction had increased over time, which showed the difference between the levels of days 24 and 27 it was clear that probiotic action against the pathogen was quite significant compared to the first day($p<0.05$). However, the control group did not have significant changes in liver status during 27 days ($p<0.05$) (Fig. 2).

Aspartate aminotransferase enzyme is also presented that the use of probiotics can improve liver function under normal conditions ($p<0.05$).

The presence of probiotics along with the pathogen indicates that probiotics can prevent very large changes in liver function, which is significantly different from the group that was exposed to the pathogen alone($p<0.05$).

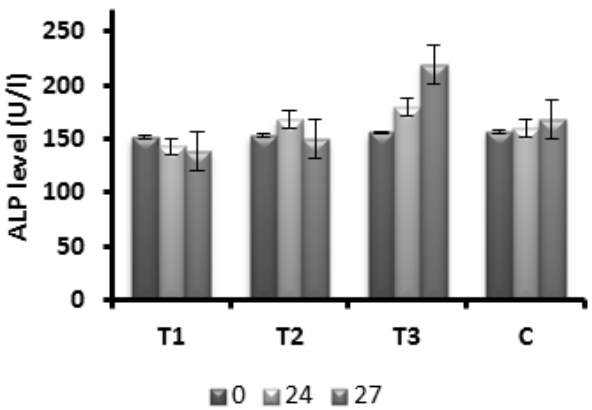

Figure 1: The changes in ALP serum level. The data were analyzed Average±2SD Error bars on the columns indicate statistical difference by Tukey's test ($p<0.05$) for the changes in ALP serum level. ALP: Alkaline phosphatase, T1 group fed with normal diet and Probiotic, T2 fed with normal diet and pathogen, T3 fed with normal diet in combination with both probiotic and pathogen, C stands for Control group just fed with normal diet

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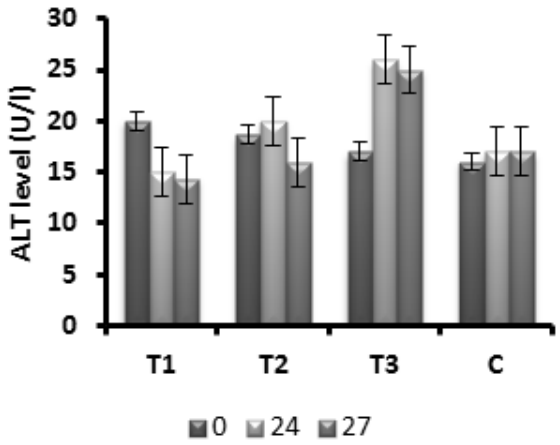

Figure 2: ALT values are expressed as mean±2SD for days 0, 24 and 27. Error bars on the columns indicate statistical difference by Tukey's test (p<0.05) for the changes in ALT serum level. Alt: Alanine aminotransferase, T1 group fed with normal diet and Probiotic, T2 fed with normal diet and pathogen, T3 fed with normal diet in combination with both probiotic and pathogen, C stands for Control group just fed with normal diet.

Aspartate aminotransferase enzyme is also presented that the use of probiotics can improve liver function under normal conditions ($p<0.05$). The presence of probiotics along with the pathogen indicates that probiotics can prevent very large changes in liver function, which is significantly different from the group that was exposed to the pathogen alone ($p<0.05$) (Fig. 3).

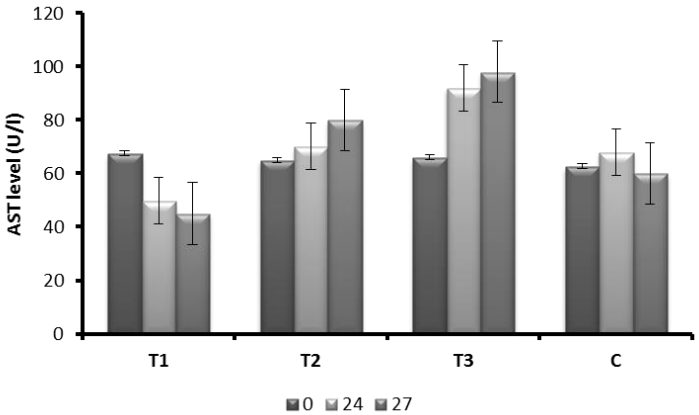

Figure 3: AST values are expressed as mean±2SD for days 0, 24 and 27. Error bars on the columns indicate statistical difference by Tukey's test (p<0.05) for the changes in ALT serum level. AST: Aspartate aminotransferase, T1 group fed with normal diet and Probiotic, T2 fed with normal diet and pathogen, T3 fed with normal diet in combination with both probiotic and pathogen, C stands for Control group just fed with normal diet.

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Discussion

Extensive use of antibiotics in aquaculture and fish farming can seriously cause antibiotic resistance of bacteria and pathogens in the environment.

For this reason, it is quite clear that we must look for an alternative way to prevent the use of antibiotics or reduce them in food sources and the environment as well. A similar study by Adorian *et al*. In 2019 showed that feeding *Bacillus subtilis* and *B. Lishiniformis* as probiotics reduced liver enzyme activity (AST, ALT, and ALP) in fish, which was consistent with findings of the present study, but with different results in the study of (Abdel-Moneim *et al*., 2020) *Bacillus subtilis*, increased the activity of liver enzymes in Carp, which is in contradiction with the results of the present study (Young *et al*., 2021). Also stated in 2021 that the use of *Lactobacillus acidophilus*, *Streptococcus thermophilus*, *Bifidobacterium bifidum* and the yeast *Saccharomyces cerevisiae* as a single probiotic affected serum AST levels while ALT did not change significantly.

Regarding the effect of exposure to the pathogen on liver enzymes, (Vignesh *et al*., 2019) stated that *Aeromonas hydrophila* infection can increase ALT and AST blood level, which was also observed in the present study in the presence of Salmonella. The difference was that the increase in liver enzyme activity in the groups receiving the probiotic diet was significantly less than the group receiving the basal diet.

As previously proven, probiotic nutrition with pathogen inhibition (Zhang *et al*., 2018) and reduction of liver enzymes AST, ALT, ALP serum (Adorian *et al*., 2019) is effective in reducing the complications of pathogenicity of infectious agents and damage to internal organs of fish. According to the results of the *Lactobacillus casei* probiotic test, it can be considered as a valuable supplement in the prevention and control of complications caused by *Salmonella typhimurium* pathogenesis in the liver as a very important organ in the immune system of valuable ornamental Koi fish.

In the present study, the probiotic *Lactobacillus casei* was used as a dietary supplement for Koi fish exposed to the pathogen *Salmonella typhimurium*. The effect of this probiotic bacterium on the liver function of Koi fish in the face of the pathogen was investigated. To evaluate liver function, three important enzymes of Alkaline phosphatase, Aspartate aminotransferase and Alanine aminotransferase were measured. Samples freshly taken from the tail end of the fish were tested by biochemical enzymatic methods. Based on the results, the effect of *Lactobacillus casei* probiotic in comparison with the control group on the improvement of liver function in this species of fish was investigated in such a way that the presence of probiotics alone may cause liver function in fish under normal or optimized conditions. However, the group that was not exposed to any probiotic agents showed a clear increase in the level of Alkaline phosphatase in the liver, which indicates the destruction of liver cells.

Regarding the results of Alanine aminotransferase levels in comparison with the control group and other groups, as

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well, it can be argued that the fish were exposed to the probiotic factor alone experienced optimal liver function in terms of liver status. However, in the group exposed to the pathogen along with the probiotic agent, the pathogen first increased the amount of Alanine aminotransferase enzyme. But finally, with the action of probiotic factors, a decrease in enzyme levels in fish can be seen. In the third group, which did not use any probiotics, to increase the level of Alanine aminotransferase in fish is observed significantly.

The study of Aspartate aminotransferase showed that the use of probiotics can improve liver function, which in fact with a behavior similar to the enzyme Alanine aminotransferase. Based on the results of the present study, the presence of probiotics in the diet of fish can improve liver function, which is evident in the study of two enzymes ALT and AST. The simultaneous presence of pathogens and probiotics can cause a slight increase in the two enzymes ALP and ALT, which can be due to the response of hepatocytes to the pathogen. However, this process progresses to recovery after day 24, which indicates an improvement in liver function. It should be noted that this issue has no statistical significance in the presented graphs.

The most significant changes were observed in the group that had a normal diet and were exposed to the pathogen. In group T3, the presence of a pathogen without the presence of a contributing factor to the liver indicates that the condition of the liver deteriorates on the 24th and 27th day of the test with a significant difference compared to the day of onset.

In the study of three main indicators of liver diagnosis, it was found that one of the proposed ways to have healthy fish both from the point of view of aquaculture and as an animal model, using probiotics such as *Lactobacillus casei* can significantly improve liver and therefore metabolic function.

References

Adorian, T.J., Jamali, H., Farsani, H.G., Darvishi, P., Hasanpour, S., Bagheri, T. and Roozbehfar, R., 2019. Effects of probiotic bacteria Bacillus on growth performance, digestive enzyme activity, and hematological parameters of Asian sea bass, *Lates calcarifer* (Bloch). *Probiotics and Antimicrobial Proteins*, 11(1), 248-255.

Qin, C., Xie, Y., Wang, Y., Li, S., Ran, C., He, S. and Zhou, Z., 2018. Impact of Lactobacillus casei BL23 on the host transcriptome, growth and disease resistance in larval zebrafish. Frontiers in Physiology, 9,.1245.

Zhang, D.X., Kang, Y.H., Chen, L., Siddiqui, S.A., Wang, C.F., Qian, A.D. and Shan, X.F., 2018. Oral immunization with recombinant Lactobacillus casei expressing OmpAI confers protection against *Aeromonas veronii* challenge in common carp, Cyprinus carpio. *Fish & Shellfish Immunology*, 72, 552-563.

Moneim, A., Eid, M.A., Selim. A.D., Basuony, A.H., Sabic, M.E., Ahmed A Saleh, A.A. and Tarek A Ebeid, A.T., 2020. Effect of Dietary Supplementation of Bacillus Subtilis Spores on Growth

[DOI: 10.18331/SFS2021.8.1.9]

Performance, Oxidative Status, and Digestive Enzyme Activities in Japanese Quail Birds. *Tropical Animal Health and Production*, 52(2), 671-80.

Hasan, K.N. and Banerjee, G., 2020. Recent Studies on Probiotics as Beneficial Mediator in Aquaculture: A Review. *The Journal of Basic and Applied Zoology*, 81(1), 1-16.

Young, N.L., Shin, J.M., Youn, S.G., Yoon, J.S., Choi, R.Y., Kim, S.H., Gupta, H., Han, H.S., Kim, K.B. and Lee, Y.D., 2021. Lactobacillus Attenuates Progression of Nonalcoholic Fatty Liver Disease by Lowering Cholesterol and Steatosis. *Clinical and Molecular Hepatology*, 27(1), 110.

Huanli, L., Whitehouse, A.C. and Baoguang, Li., 2018. Presence and Persistence of Salmonella in Water: The Impact on Microbial Quality of Water and Food Safety. *Frontiers in Public Health*, 6, 159.ta

Mahdavi, S. and Isazadeh, A., 2019. Lactobacillus Casei Suppresses Hfq Gene Expression in Escherichia Coli O157: H7. *British Journal of Biomedical Science*, 76(2), 92-94.

Peterson, L. and Kaur, P., 2018. Antibiotic Resistance Mechanisms in Bacteria: Relationships between Resistance Determinants of Antibiotic Producers, Environmental Bacteria, and Clinical Pathogens. *Frontiers in Microbiology*, 9, 2928.

Roope, L.S.J., Smith, D.R., Pouwels, B.K., Buchanan, J., Abel, L., Eibich, P., Butler, C.C., Tan, S.P, Walker, S.A. and Robotham, V.J., 2019. The Challenge of Antimicrobial Resistance: What Economics Can Contribute. Science, 364(6435).

Vignesh, S., Velu, V. and Uthandakalaipandiyan, R., 2019. Determination of Lethal Dose of *Aeromonas hydrophila* Ah17 Strain in Snake Head Fish Channa Striata. *Microbial Pathogenesis*, 127, 7-11.

Tachibana, L., Telli, S.G., Dias, C.D.D., Gonçalves, S.G., Ishikawa, M.C., Cavalcante, B.R., Natori, M.M., Hamed, B.S. and Ranzani-Paiva, T.J.M., 2020. Effect of Feeding Strategy of Probiotic Enterococcus Faecium on Growth Performance, Hematologic, Biochemical Parameters and Non-Specific Immune Response of Nile Tilapia. Aquaculture Reports, 16, 100277.

Yim, M.W., Forsythe, J.S. and El-Nezami, H., 2019. Probiotics Interaction with Foodborne Pathogens: A Potential Alternative to Antibiotics and Future Challenges. *Critical Reviews in Food Science and Nutrition*, 59(20), 3320-33.

Wanka, Konrad M., Damerau, T., Costas, B., Krueger, A., Schulz, C. and Wuertz, S., 2018. Isolation and Characterization of Native Probiotics for Fish Farming. *Bmc Microbiology*, 18(1), 1-13.

Zhang, C.M., Xu, M.L., Xiao Mou, M.X., Xu, H., Liu, J., Miao, H.Y., Wang, C.X. and Li, X., 2019. Characterization and Evolution of Antibiotic Resistance of Salmonella in Municipal Wastewater Treatment Plants. *Journal of Environmental Management*, 251, 109547.

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