

Investigation of the effects of *Bifidobacterium bifidum* as a probiotic on liver function enzymes due to exposure to *E.coli* O157H7 in Koi fish (*Cyprinus rubrofuscus*)

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Abstract

Under intensive aquaculture conditions, fish are exposed to many pathogens, due to that strengthening their immune system is an alternative significant way to reduce mortality rate and disease-related complications. Probiotics are valuable oral nutrition supplements for this purpose. In the present investigation, there were used Koi fish (n=120) with the characteristics of 10±3 Cm in length and an average of 20±1 g in weight classified in 4 groups under 2 replication. The classification of groups was as follow: T1; 24-day treated with the probiotic diet of *Bifidobacterium bifidum* (1.5×10⁸ CFU/ mL), T2; 24-day treated with the probiotic diet of *Bifidobacterium bifidum* and 72 hours of exposure to *E.coli* O157H7 pathogen (1.5×10⁸ CFU/ mL), Sham group; 24-day probiotic-free commercial feed treatment and 72 hours of exposure to *E.coli* O157H7, and control group (C); which had fed neither the probiotics nor any pathogens. In the present scientific investigation, the feeding processes were done twice a day at 10:00 a.m. and 2:00 p.m. where the samples were stored at 12:12 cycle of dark and light. ALT, AST and ALP serum levels were examined to determine the symptoms of disease caused by the pathogen. These liver function tests (LFT) were examined on days 24 and 27. As a conclusion, it is found that probiotic complements feeding cause to decrease the LFT level compared with the control group on days of 24 and 27 ($p<0.05$). Also, in the probiotic-fed group, after exposure to the pathogen, the level of serum LFT increased compared with the control group ($p<0.05$), the probiotic utilization as a supplement reveals the better efficiency of the liver during ageing, as well ($p<0.05$).

Keywords: *Bifidobacterium bifidum*, *E.coli* O157H7, Koi fish, *Cyprinus Rubrofuscus* Alanine aminotransferase, Aspartate aminotransferase, Alkaline phosphatase.

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Introduction

Escherichia coli (ETEC) is a food- and water-borne pathogen that is responsible for hundreds of millions of diarrhea cases worldwide in travelers (i.e., traveler's diarrhea) and infants under the age of 5 years in poor countries (Roussel *et al.*, 2018). One of the ways of transmitting is contact with infected fish and according to reports *Escherichia coli* was present in the fish body and their secondary products, which may be harmful to human health (Fapohunda *et al.* 1994). Because some human pathogens, such as E.coli, have the potential to grow and reproduce in the intestines, mucus, and tissues of fish. The main cause of such an infection could be the utilization of wastewater in aquaculture pools. Fish has the potential to be a long-term carrier and host for some human infection pathogens (Ampofo and Clerk, 2010). Traditional infectious disease control strategies using antibiotics and chemical antibiotics are not recommended due to the emergence of bacterial resistance and concerns about environmental impacts and necessity of protection of wildlife in nature (Esiobu *et al.*, 2002; Bachère, 2003) and using the methods that can be alternative to antibiotics has been widely under attention and is a recommendation by the European Union and the World Health Organization (CDC, 2014). However, because there are no commercial compounds available for prevention, the use of probiotic microorganisms, as an alternative method, has provided a broad field for research in this area (Roussel *et al.*,

2017). Probiotics are living microorganisms that, if consumed in adequate quantities, can boost the host's health (Hill *et al.*, 2014).

The most successful and biocompatible treatment way has been achieved by using immune system stimulation as an environment-friendly approach of disease management and control and one of these immunomodulatory complements are probiotic bacteria that can increase fish resistance against pathogens and disease by improving their immune system function and water quality (Wang *et al.*, 2008). Because Immune systems function enhancement has been proposed as an alternative method for the prevention and control of various diseases (Merrifield *et al.*, 2010; Nayak, 2010; Ringo *et al.*, 2010). Therefore the use of probiotic bacteria to control potential pathogens is an alternative method that is gaining acceptance within the aquaculture industry (Gomez – Gil *et al.*, 2000).

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 acclimatise fishes, they were transferred to Razef Research Complex located in Islamic Azad University, Science and Research branch, Shahriar-Iran .

Both *Bifidobacterium bifidum* and E.coli O157: H7 of PTCC 12900 strain was used in the current research. *E.coli*

O157: seem very old H7 were supplied in lyophilized form from the Faculty of Veterinary Medicine of Tehran University of Veterinary Medical Sciences. To confirm the bacterial strain and characteristics approval microscopic examination and biochemical tests were performed. *Bifidobacterium bifidum* was prepared in lyophilized form from the microbial bank of Iran Biological Resources Center, cultured in Man Rogosa and Sharp (MRS) broth (Merk, Germany), and incubated at 37°C (160 rpm) in anaerobic condition for 24-48 hours. Then cell-free supernatants (CFS) were isolated based on optical density (OD) (Sahandi *et al.*, 2016). The desired cell density was prepared using a spectrophotometer (Libera model S22: Biochrom, Cambridge, the UK) at 610 nm, and the desired colony-forming unit (CFU) (1.5×10^8 CFU/mL) was obtained using serial dilution (Sahandi *et al.*, 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 *E.coli*, T2; the Probiotic diet with exposure to *E.Coli*, T3; feeding with basal diet and exposed to *E.Coli* and the control group which was fed with the basal diet with no exposure to *E.Coli*.

The diets were arranged based on 5% of the bodyweight of fish, 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. (Wang *et al.* 2016). At the end of the feeding period, T2 and T3 groups were exposed to *E. coli* for 72 hours (Pan *et al.*, 2013).

Sampling was performed to evaluate liver enzymes in the first days, after the feeding period and after the end of exposure, as well. All samples were gathered by routine blood sampling method from the caudal vein. The samples prepared for biochemical tests were immediately transferred to the laboratory in an ice pack container and all tests were performed using the commercial kit of Pars Azmoon Company.

Results

The results obtained from ALP are shown in Figure 1. Based on these results on day 24 both of T1 and T2 groups revealed a decrease ($p > 0.05$) While T3 showed no significant change ($p > 0.05$) in ALP level. Also on the 27th day after exposure to the pathogen, T2 decreased and T3 increased compared to the control group ($p < 0.05$).

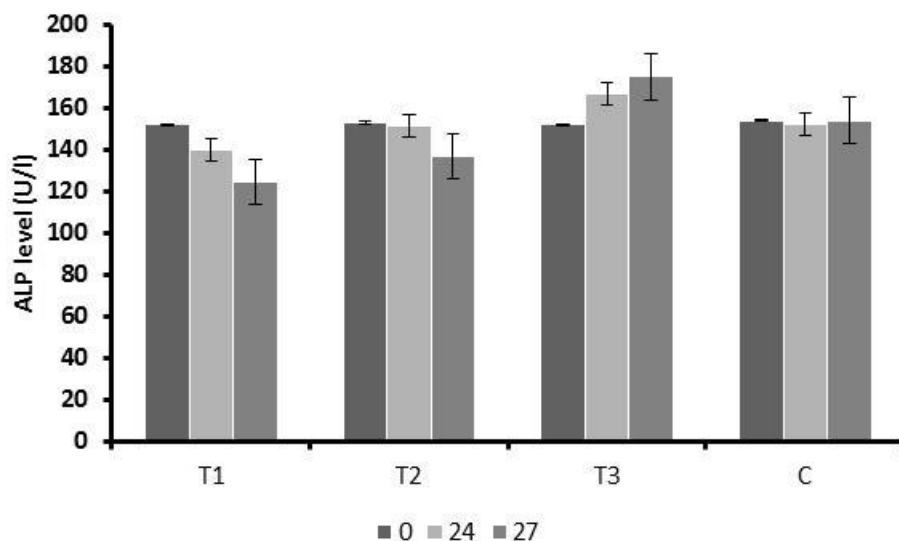


Figure 1: The changes in ALP serum level. The data were analyzed Average \pm 2SD Error bars on the columns indicate statistical difference by Tukey’s test ($p<0.05$) for the changes in ALP serum level. ALP: Alkaline phosphatase, T1 group fed with normal diet and Probiotic, T2 fed with normal diet and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

Examination of the ALT enzyme on day 24, did not show any significant change in the groups ($p>0.05$). But on day 27, a significant decrease ($p<0.05$)

in T1 and T2 and a significant increase in T3 ($p<0.05$) compared to the control group was observed (Fig. 2).

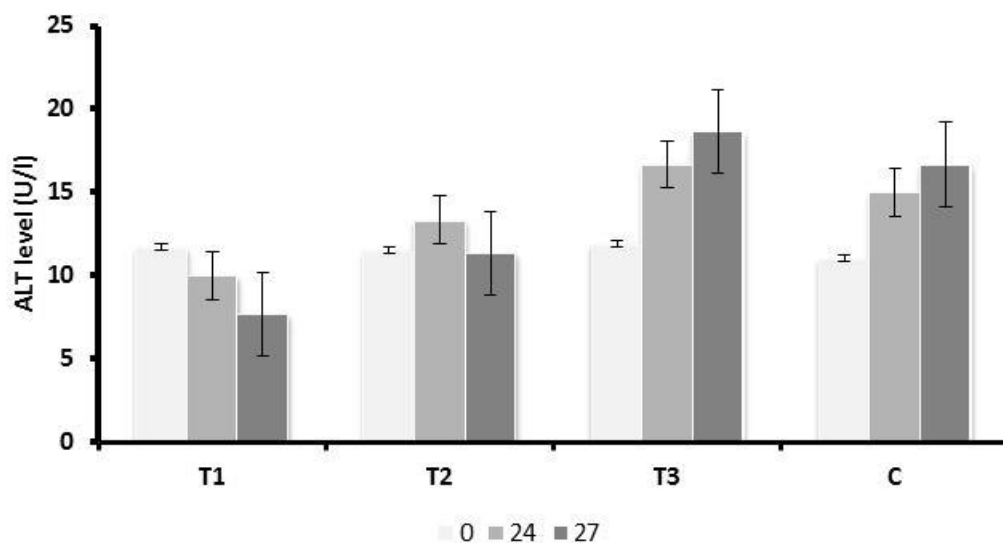


Figure 2: ALT values are expressed as mean \pm 2SD for days 0, 24 and 27. Error bars on the columns indicate statistical difference by Tukey’s test ($p<0.05$) for the changes in ALT serum level. Alt: Alanine aminotransferase, T1 group fed with normal diet and Probiotic, T2 fed with normal diet and pathogen,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

The results obtained from AST are shown in Figure 3. Based on these results on day 24 both of T1 and T2 groups revealed a decrease ($p < 0.05$) While T3 showed no significant change

($p > 0.05$) in AST level. Also on the 27th day after exposure to the pathogen, T2 decreased and T3 increased compared to the control group ($p < 0.05$).

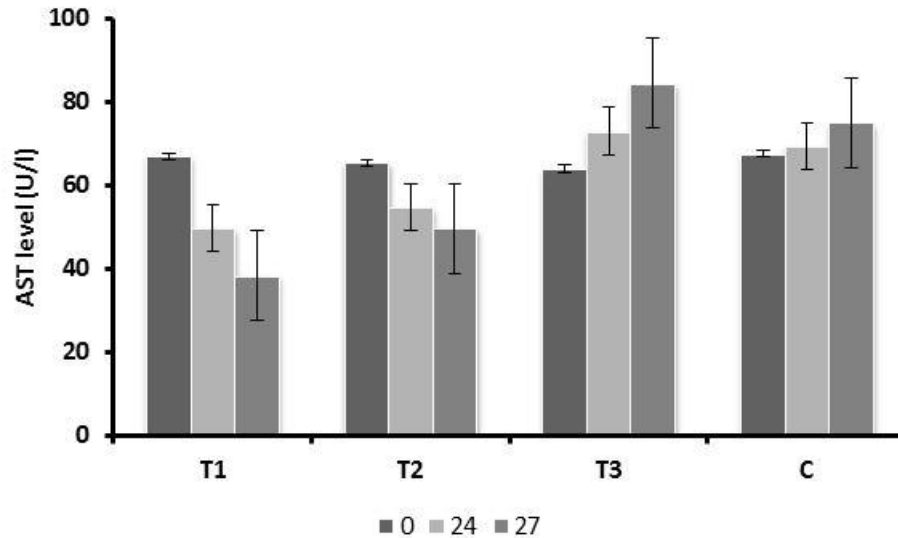


Figure 3: AST values are expressed as mean \pm 2SD for days 0, 24 and 27. Error bars on the columns indicate statistical difference by Tukey's test ($p < 0.05$) for the changes in ALT serum level. AST: Aspartate aminotransferase, T1 group fed with normal diet and Probiotic, T2 fed with normal diet and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

Discussion

Fish are at risk for many infectious agents under conditions of intensive aquaculture, and chemical compounds, such as antibiotics, which are used as chemical treatments. In this regard, probiotics are among the best supplements that have been identified as a preventive alternative a suitable replacement for antibiotics (Wan *et al.*, 2019). One of the most important and reliable indicators in assessing the health status of fish is the measurement of blood factors. Therefore, to compare the effect of different diets on health and defense system, blood indicators can be examined (Liu *et al.*, 2019).

Elevated levels of ALT, AST and ALP in liver serum indicate liver damage. According to probiotic nutrition studies, it is revealed that this alternative medication can reduce the liver enzymes in the serum level of fish (Adorian *et al.*, 2019) as observed in the present study. Also, a similar study by Adorian *et al.*, showed that feeding *Bacillus subtilis* and *Lishiniformis* as probiotic supplements could reduce liver enzymes (AST, ALT and ALP) in Asian sea bass (Adorian *et al.*, 2019) Despite the differences in probiotic species and fish species, it is consistent with the results of our study.

On the other hand in different results, no significant change in serum ALT and AST levels were observed in the study of

Safari *et al.* where they used *Enterococcus* probiotics in rainbow trout (Safari *et al.*, 2016). Also, serum ALP levels in black amur fish, which were fed with the synbiotic bacterium *Bacillusformis* and fructooligosaccharides revealed significantly increased in comparison to the control group (Zhang *et al.*, 2013). And Kane *et al.* (2016) Studies have shown the use of bacteria *Lactobacillus plantarum* increased serum ALP activity after consumption of probiotics, this difference is probably due to differences in the type of probiotic, fish species and feeding duration.

According to previous studies, exposure to bacterial pathogens leads to an increase in liver enzymes (Park *et al.*, 2013; Minemura *et al.*, 2014). In this regard, according to the report of Wang *et al.* (2017), infection with *Aeromonas hydrophila* increased the level of liver enzymes in carp, which is correlated with the results of the current study. This is in line with the results of the present study, but the remarkable point is the changes observed in the groups fed with probiotics.

The findings in this study show a reduction in liver damage and serum levels of liver enzymes in fish fed probiotics diet in exposure to bacterial infections. In this regard and with similar results Ghanei-Motlagh *et al.* (2021) showed that feeding with a probiotic diet reduces the level of liver enzymes in Asian seabass in the face of *Vibrio harveyi*.

Conclusion

Based on the results obtained in this study, it can be concluded that adding *Bifidobacterium bifidum* probiotic in the diet of Koi ornamental fish could improve their liver functional enzymes, especially in exposure to *E. coli* O157H7 intestinal pathogen. In addition, *B. bifidum* probiotic is recommended as an effective supplement to boost the immune system in koi fish.

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