The nutritional effect of probiotic *Lactobacillus acidophilus* on intestinal microbial flora and inhibition of Shiga toxin (Stx2) gene expression in zebrafish (*Danio rerio*) exposed *to Escherichia coli* O157:H7

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Received: January 2022 Accepted: May 2022

Abstract

Nowadays, the use of probiotics is considered an alternative solution instead of using antibiotics. This study aimed to investigate the nutritional effect of probiotic *Lactobacillus acidophilus* on intestinal microbial flora and inhibition of Shiga toxin (Stx2) gene expression in zebrafish exposed *to Escherichia coli* O157:H7. For this purpose, 600 zebra fish were divided into four groups with three repetitions including feeding with a commercial diet without probiotics (C and T3) and feeding with a diet containing probiotics with a concentration of 1.5×10^{8} cfu ml^{-1} (T1 and T2). The fish were fed for 24 days. Then T2 and T3 groups were exposed to *E. coli* O157: H7 for 72 hours. Sampling was done on days 0, 14, 24, and 28. The obtained results showed that feeding with probiotics led to an increase in the growth of lactobacilli in the intestine ($p<0.05$). Also, *L. acidophilus* decreased the expression of the Stx2 gene in fish exposed to the pathogen ($p<0.05$). Therefore, *L. acidophilus* used in the diet improved intestinal microbial flora and inhibited toxin production in zebrafish exposed to *E. coli* O157: H7. This pathogen is very important in terms of prevalence, nutritional role, and the effect of intestinal microbial flora in reducing the pathogenicity *of E. coli*. In addition, zebrafish is an animal model to investigate the pathogenicity of human pathogens. Therefore, the results obtained can be generalized to humans.

Keywords: *Lactobacillus acidophilus*, *Escherichia coli O157: H7*, Shiga toxin, Zebrafish

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Introduction

Today, the epidemic of microbial diseases and resistance to antibiotics has increased the need to identify alternative compounds. Probiotics are compounds that have been investigated by researchers as an alternative to antibiotics in recent decades. Probiotics are live microorganisms that provide health benefits to the host with adequate administration (María Remes-Troche *et al.*, 2020). These compounds are widely used in the aquaculture industry. These probiotics modulate the microbial flora (Hoseinifar *et al.*, 2015), improve the intestinal structure (Alavinezhad *et al.*, 2020), improve immune function (Kazempour and Kazempoor, 2022), and increase resistance to pathogens (Elahi *et al.*, 2020; Mollanourozi *et al.*, 2021) in aquatic animals.

Lactobacilli are microorganisms that are mainly used as probiotics in nutrition (María Remes-Troche *et al.*, 2020). Lactic acid bacteria (LAB) are facultatively anaerobic, catalase-negative, gram-positive, and non-sporulating bacilli (Goldstein *et al.*, 2015). These bacteria can resist the pH of the digestive tract and bile. They also can bind to mucus, inhibit the growth of other bacteria, and modulate the immune system (María Remes-Troche *et al.*, 2020).

Bacillus acidophilus was first isolated from infant feces in 1900 by Moro. This bacterium is a short, gram-positive, obligate homofermentative bacillus. This bacterium can grow optimally at temperatures of 37 to 42 ^{O}C and pH 5.5 to 6.0. It is also the least tolerant of LABs to oxygen deficiency (Bull *et al.*, 2013). Hoseinifar *et al.* (2015) reported the effect of feeding with *L. acidophilus* on improving microbial flora, immune parameters, and increasing stress resistance in zebrafish. Elahi *et al.* (2020) observed a reduction in complications caused by exposure to an invading intestinal pathogen after feeding with *L. acidophilus* in zebrafish.

Escherichia coli is a rod-shaped, gram-negative, facultatively anaerobic bacterium that was first described by Theodor Escherich in 1885. Most of the *E. coli* strains are the natural flora of the digestive tract of humans and animals. But some strains have become pathogenic with the help of virulence factors (Kaper *et al.*, 2004).

E. coli O157:H7 is one of the pathogenic strains. Its main virulence factors include Shiga toxins (Stx), production of pathogenicity island called enterocyte elimination site, and production of F-like plasmid pO157 (Lim *et al.*, 2010). Producer Strains of Stx2 are more involved in severe disease complications than Stx1 (Boerlin *et al.*, 1999). Although the pathogenicity of *E. coli* O157:H7 is often discussed in humans, previous studies have reported the occurrence of disturbances in physiological functions in fish exposed to this bacterium (Elahi *et al.*, 2020; Loghmani *et al.*, 2022).

Zebrafish (*Danio rerio*) is used to investigate the stages of development using techniques such as fluorescent tracer due to its characteristics such as abundant egg production per week,

small size, easy maintenance, and transparency of the embryo. This aquatic organism is an excellent organism for biological investigations in vertebrates (Arayesh *et al.*, 2021). Therefore, it is common to use zebrafish to investigate the pathogenicity of bacterial pathogens in vertebrates, including humans and fish (Nowik *et al.*, 2015). This study aimed to investigate the nutritional effect of probiotic *Lactobacillus acidophilus* on intestinal microbial flora and inhibition of Shiga toxin (Stx2) gene expression in zebrafish exposed *to Escherichia coli* O157:H7.

Materials and methods

Experimental fish and rearing

Zebrafish (average weight: 0.13±0.1 g) were purchased from a local aquaculture center. These fishes were kept for two weeks in the laboratory in glass aquariums with an aeration system for adaptation. The fish were fed a commercial diet (BioMar, France) twice a day (2% of body weight). Feces and discarded feed were siphoned daily. After the adaptation period, the fish were divided into two control groups (fed with a commercial diet without probiotics, C and T3) and two groups of diets containing probiotics with a concentration of $1.5x10^{8}$ cfu mL^{-1} (T1 and T2) (with three replications). They were divided into 12 aquariums (50 fish in each aquarium). The fish were fed with diets for 24 days. Then the fish of the T2 and T3 groups were exposed to *E. coli* O157: H7 for 72 hours. The basic water quality parameters including temperature (26±1.0 °C), pH (7±0.4), dissolved oxygen concentration (6.9±0.5 mg L^{-1} 10^{5}), and photoperiod (14L:10D) were maintained during the experimental period and 30 % of aquarium water was changed daily.

Culture of probiotic bacteria

Lactobacillus acidophilus La-5 was prepared in lyophilized form from the Iranian National Center for Genetic and Biologic Resources. Then bacteria were injected into deMan, Rogosa, and Sharpe (MRS) agar plates (Merc, Germany). The bacterium was incubated in a low aerobic condition for 48 hours in a CO2 incubator with a temperature of 35 °C and 150 rpm until reaching the appropriate concentration. The desired bacterial dilution (1.5×10^{8} cfu mL^{-1}) was prepared using serial dilution (Elahi *et al.*, 2020).

Preparation of experimental feed

A probiotic diet ration was prepared by adding *L. acidophilus* suspension (1.5×10^{8} cfu mL^{-1}) to commercial feed (BioMar, France). It was incubated in ice for 15 minutes to absorb the bacteria. Also, fish feeding with a basic ration was prepared by combining commercial feed with sterile PBS (in the volume equivalent to bacterial suspension) (Wang *et al.*, 2016). The ration was prepared daily and feeding was done twice a day based on 2% of fish body weight.

Challenge test with E. coli O157: H7

E. coli O157: H7 PTCC 1338 was prepared in lyophilized form from the

Iranian National Center for Genetic and Biologic Resources. Bacteria were grown in Luria-Bertani (LB) broth (Sigma Aldrich) at 37°C at 200 rpm overnight. Then the desired bacterial dilution (1.5×10^{8} cfu mL^{-1}) was prepared using serial dilution (Mohsin *et al.*, 2015). After feeding with rations for 24 days, fish in T2 and T3 groups were exposed to *E. coli O157: H7* for 72 hours. The tanks' water was not changed during the exposure time and feeding was not done.

Intestinal microbiota analysis

This part of the experiment was performed based on the method of Hoseinifar *et al.* (2015) with some changes. Three fish were randomly collected from each aquarium on days 0, 14, 24, and 28. Then, the intestines of the fishes were carefully separated and homogenized under sterile conditions (the samples from each aquarium were combined). 100 mL of homogenized samples were cultured on an MRS agar medium. The plates were incubated for 5 days at room temperature (25°C). Finally, countable plates (with 30 to 300 colonies) were calculated based on the CFU/g unit and the results were reported.

Total RNA extraction and cDNA synthesis

Three fish from each aquarium were randomly isolated for Stx2 extraction on days 0, 14, 24, and 28. The intestinal contents of the samples were isolated under sterile conditions and homogenized in 1 mL sterile saline solution (Asahara *et al.*, 2004). Silica gel-based membrane and RNeasy Mini Kit (Qiagen, Mississauga, Ontario Canada) were used for total RNA isolation. 2 ml of the sample was incubated with two volumes of RNA Bacteria Protect™ (Qiagen) at room temperature for 5 minutes. Then the sample was centrifuged for 5 minutes (750g) at 4°C and the supernatant was centrifuged again for 10 minutes (5000g) at 4°C. The obtained mixture was incubated in 100 µL of lysozyme (10 mg/mL) in TE buffer (pH 8.0) and 20 µL of proteinase K (20 mg/mL) at room temperature for 10 minutes. Then this mixture was vortexed and centrifuged in 350 µl of RLT buffer containing 2% beta-mercaptoethanol. The supernatant was separated and mixed with 100% ethanol, and the lysate was applied directly to the RNeasy mini-column. Washing was done twice with 700 µL of RW1 buffer and once with 500 µL of RPE buffer. Total RNA was washed from the column with 45 µL of RNase-free water after removing the residual ethanol by centrifugation. After RNA preparation, genomic DNA contamination was removed using the DNA-free™ kit (Ambion, Cambridge, UK) according to the manufacturer's instructions. Then, the total RNA concentration was determined by the spectrophotometric method. Reverse transcription of RNA was performed using SuperScript II (Invitrogen) according to the manufacturer's instructions. cDNA synthesis was performed to inactivate the enzyme under temperature

conditions of 65°C for 5 min, 25°C for 2 min, 25°C for 10 min, 42°C for 50 min, and 70°C for 15 min (Carey *et al.*, 2009).

Real-time PCR

25 µL of the solution containing 2 µL of reverse-transcribed cDNA, 12.5 µL of Cyber Green PCR Power Master Mix (containing Cyber Green I, Amplitag DNA polymerase, dNTP, ROX inactive dye, and optimized buffer), U0.25 AmpErase® Uracil N-glycosylase (UNG; Applied Biosystems), 500 nM of each primer and nuclease-free water were used for each Real-time PCR reaction. The sequences of the primers used were designed using Primer Express 2.0 software (Applied Biosystems) (Table 1). Primer specificity was determined by melting curve analysis and its efficiency was determined by cDNA serial dilution Power SYBR® Green. Amplification and detection were performed on the Multiplex Quantitative PCR system with an initial temperature of 50°C for 2 minutes. Deactivation of the AmpErase UNG enzyme and activation of AmpliTaq DNA was performed at 95°C for 10 minutes. Also, 40 cycles of amplification were performed at 95°C for 15 s, 63°C for 1 min, and 72 °C for 30 s. The next melting curve check was 95°C for 1 min, cooling to 55°C and then heating slowly to 95°C. Fluorescence data were collected at the end of each cycle. mRNA levels were measured using the 2-ΔΔCT method according to User Bulletin No. 2 (P/N 4303859) (Carey *et al.*, 2009).

Table 1: PCR primers used in real-time PCR gene expression assays (Carey *et al.*, 2009).

Primer	Size (bp)	Sequence (5' - 3')
Stx 2	184	F: TTGCTGTGGATATACGAGGGC R: TCCGTTGTCATGGAAACCG

Statistical analysis

The normality of data and homogeneity of variance were checked at first. Data analysis and mean comparison were performed by one-way analysis of variance (ANOVA) and Duncan's multiple range tests, respectively. Statistical analyzes were performed using SPSS version 18.0 statistical software (SPSS Inc., Chicago, IL, USA).

Results

Intestinal microbiota

The results of Lactobacillus colonization in the intestines of zebrafish as a result of feeding with *L. acidophilus* and exposure to *E. coli* O157:H7 are shown in Table 2. Based on the obtained results, no significant difference was observed between the groups on day zero ($p>0.05$). Lactobacillus colonization in T1 and T2 increased significantly on days 14 and 24 compared to C and T3 ($p<0.05$). The results of the 28th day showed a significant decrease in the T2 and T3 groups. Also, the lowest value was

recorded in T3 and the highest value was recorded in T1 (p<0.05).

Table 2: Lactobacillus colonization in the intestines of zebrafish fed with *L. acidophilus* and exposed to *E. coli O157: H7* on different days. The results are shown as mean ± standard deviation.

Treatment	Day 0	Day 14	Day 24	Day 28
C	6.09±0.02 [a]	6.11±0.03 [b]	6.2±0.02 [b]	6.14±0.04 [b]
T1	6.11±0.04 [a]	7.3±0.04 [a]	8.46±0.05 [a]	8.47±0.07 [a]
T2	6.1±0.01 [a]	7.38±0.05 [a]	8.51±0.05 [a]	5.47±0.05 [c]
T3	6.11±0.01 [a]	6.14±0.02 [b]	6.2±0.04 [b]	3.47±0.07 [d]

Different lowercase letters in each column indicate significant differences between treatments (p<0.05).

Shiga-toxin gene expression

The stx2 gene expression was checked on days 0, 14, and 24 of feeding with *L. acidophilus* and after 72 hours of exposure to *E. coli O157: H7* (day 28) by the Real-Time PCR method. Based on the obtained results, stx2 gene expression was not reported in any of the groups on days 0, 14, and 24. Also, stx2 gene expression was not observed in groups C and T1 on day 28. The highest level of gene expression was recorded in the T3 group on day 28. The results showed a significant decrease in stx2 gene expression in the T2 group compared to T3 (p<0.05) (Figure 1).

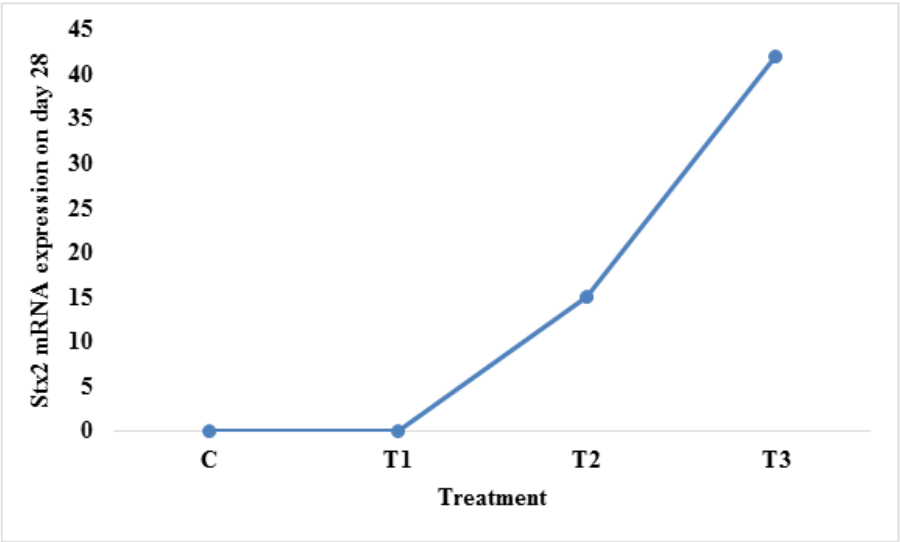

Figure 1: Inhibition of Stx2 gene expression in probiotic-fed zebrafish exposed to *E. coli* O157:H7 on day 28 of the experiment.

Discussion

Nowadays, the *E. coli O157: H7* serotype has attracted a lot of attention due to its severe pathogenicity and widespread epidemics. The increase in the secretion of Shiga toxin (Stx) after

exposure to antimicrobial agents has increased concerns about antibiotic resistance, which has led to the non-recommendation of antibiotic compounds in the treatment of this pathogen (Bielaszewska *et al.*, 2012). Therefore, probiotics can be proposed as an alternative treatment option (Mohsin *et al.*, 2015).

L. acidophilus has been used in several studies as a probiotic in various gastrointestinal diseases (María Remes-Troche *et al.*, 2020; Elahi *et al.*, 2020). However, the possible role of this probiotic in *E. coli* infections has rarely been investigated. Positive effects of probiotic-containing diets on aquatic health have been reported in several studies (Kazempour and Kazempoor, 2022), but there is limited information on the effects of L. acidophilus probiotics in fish. In this study, the effect of L. acidophilus probiotic on intestinal microbial flora and Shiga toxin (Stx2) gene expression in zebrafish exposed to *E. coli O157: H7* serotype was investigated.

In this study, the use of the *L. acidophilus* diet led to a significant increase of lactobacilli in the intestinal flora. LABs are not the dominant bacterial species in fish intestines, but they improve the microbial flora of fish intestines. Therefore, diets effective in increasing these bacteria will be valuable (Hoseinifar *et al.*, 2015). So far, the increase in the amount of LAB in the gut microbiota of fish fed with various probiotic bacteria (Gatesoupe, 2010; Hoseinifar *et al.*, 2016) and LAB strains has been reported (Balcázar *et al.*, 2007; Hoseinifar *et al.*, 2015). There have been few reports on the effect of feeding with L. acidophilus on the improvement of the intestinal microbial flora of cold-blooded animals. The results of the studies on black swordtail fish (*Xiphophorus helleri*) (Hoseinifar *et al.*, 2015) and marron crab (*Cherax cainii*) (Foysal *et al.*, 2020) were consistent with the results of this study. Based on the obtained results, administration of L. acidophilus in the diet has modulated the intestinal microbiota of zebrafish by beneficial communities. However, the confirmation of this hypothesis requires more detailed studies including molecular studies on the intestinal microbiota. The production of antimicrobial compounds or metabolites that inhibit the growth of microorganisms, and competition with other intestinal microbes for receptors and binding sites in the intestinal mucosa leads to an increase in LAB colonization (Hemarajata and Versalovic, 2013).

Shiga toxins are key pathogenic factors in *E.coli* O157:H7 and Stx genes are encoded in the genome of lambdoid lysogenic bacteriophages (Smith *et al.*, 2012). The obtained results showed a significant decrease in the expression of stx2 in fish fed with probiotics. Also, similar studies have reported the inhibitory effects of probiotic strains on stx2 expression in *E.coli* O157:H7 (Carey *et al.*, 2008; Mohsin *et al.*, 2015). *L. acidophilus* probiotic was used in this study. Ogawa *et al.* (2001) reported inhibition of Stx production by

Lactobacillus probiotic strains in laboratory conditions. Also, Kim *et al.* (2006) reported that *L. acidophilus* strains can reduce the virulence of Stx2 produced by *E. coli* O157:H7.

In this study, intestinal microbial flora and increased LAB levels were improved in fish fed with probiotics, therefore inhibition of Stx2 gene expression was caused by inhibition of *E. coli* growth in the intestine by microbial flora, which is consistent with the results of Mohsin *et al.* (2015), and AL-Imam and Flayyih (2020). Ogawa *et al.* (2001) reported that inhibition of Stx production is caused by lactic acid production by LAB bacteria. Carey *et al.* (2008) also reported that decreased stx2 expression was related to acetate production and decreased pH. Kim *et al.* (2008) reported that the use of *L. acidophilus* cell extract inhibited attachment to epithelial cells and biofilm formation by *E. coli O157: H7* in laboratory conditions. Medellin-Pena and Griffiths (2009) reported that molecules released by L. acidophilus affected the transcription of *E. coli* O157:H7 genes involved in colonization and quorum sensing. It has also inhibited the attachment of the pathogen to intestinal epithelial cells in laboratory conditions. These results can be caused by the mechanisms involved in the reduction of Stx production. de Sablet *et al.* (2009) reported that body microbiota reduces stx2 mRNA transcription by suppressing RecA transcription. The results showed that probiotic feeding improved the function of intestinal microbial flora, which is consistent with the results of de Sablet *et al.* (2009). In this study, genes related to RecA were not investigated, but it is recommended to investigate the effect of feeding with L. acidophilus on the expression of genes related to RecA in zebrafish exposed to *E.coli* O157:H7.

Conclusion

Laboratory results showed that feeding with L. acidophilus improved intestinal microbial flora and inhibited the expression of the Stx virulence gene in zebrafish exposed to *E.coli* O157:H7. Therefore, it has limited the infection. The reduction in Shiga toxin may be the first line of defense in preventing disease complications with *E.coli* O157:H7. The spread of antibiotic resistance and the prohibition of antibiotic use in *E.coli* infections show the value of the results in this study. However, it is recommended to investigate the L. acidophilus mechanism of action on *E.coli* O157:H7 bacteria, the inhibition of virulence genes, and the expression of other virulence genes in future studies. It is also suggested to investigate the immune factors of the host to consider the effect of feeding with *L. acidophilus* on the virulence of *E.coli* O157:H7.

Funding information

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Acknowledgements

We thank Dr. Seyedeh Shiva Alavinejad, Department of Aquatic Health and Diseases, Faculty of Veterinary Medicine, University of Tehran, for her assistance in the field.

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