Iran Red Crescent Med J. 2020 April; 22(4):e99400. doi: 10.5812/ircmj.99400.

Published online 2020 April 5. **Research Article**

Study of the Histopathologic Effects of Probiotic *Lactobacillus acidophilus* in Exposure to *E. coli O157: H7* in Zebrafish Intestine

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Received 2019 November 06; **Revised** 2020 January 19; **Accepted** 2020 February 22.

Abstract

Background: Over the past three decades, the use of probiotics has increased as growth promoters and effective supplements to reduce the pathogenicity of pathogens. In this regard, Lactobacillus bacteria are among the most common probiotics used, as they can help strengthening the digestive system and therefore reducing intestinal hystopathological damage when encountering pathogens.

Objectives: This study aimed to evaluate the histopathologic effects of *Lactobacillus acidophilus* as a dietary supplement in zebrafish ration, as an appropriate laboratory model, and in the exposure to *Escherichia coli O157: H7*.

Methods: In this project, 48 fish were grouped in 4 aquariums and monitored for 30 days; control group (*C1A1*) received basic ration; *B1A1* group received control group ration and were exposed to *E. coli O157: H7*; Treatment 1 (*T1A1*) received basic ration containing *L. acidophilus* with no exposure to *E. coliO157: H7*, and Treatment 2 (*T2A2*) received basic ration containing *L. acidophilus* with exposure to *E. coli O157: H7*. During 30 days of the experiment, the samples were taken from the intestinal tissue in the days 15, 27, and 30 for the histopathological examinations.

Results: The results of the findings showed a significant increase in the length of the intestinal villi and the number of goblet cells in the studied tissue in the group treated with a ration containing probiotic supplements compared to the control group ($P < 0.001$, $P < 0.01$, $P < 0.05$). Also, in the group exposed to *E. coli O157: H7*, histopathological changes including mild edema, inflammatory cell accumulation in the intestinal mucosal tissue, severe necrosis and epithelium loss in the intestinal tissue were evident. These symptoms were much lower in the group fed with probiotic.

Conclusions: According to the obtained data, it can be concluded that feeding fish using *L. acidophilus* supplement can produce very beneficial effects in reducing tissue damage caused by *E. coli O157: H7* infection in zebrafish intestines as a laboratory model.

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

1. Background

Probiotics have been widely studied in recent years. The usage of these compounds in the treatment and prevention of the diseases in particular gastrointestinal diseases has shown desirable results. Today, the use of probiotics as an alternative to antibiotics has become more common in coping with and immunity against intestinal pathogens (1).

Probiotics are living microorganisms that can potentially have beneficial effects on the host health when consumed by living organisms. These beneficial organisms perform a competitive role in preventing the growth of pathogenic bacteria in the digestive tract. Several types of bacteria can be considered as probiotics; among them acidic bacteria are the most commonly used probiotics. *L. acidophilus* is an important group of bacteria producing lactic acid and a probiotic. The studies have shown that colonization of this bacterium in the intestine inhibits the binding process of *E. coli O157: H7* to the intestinal epithelial cells (2).

Escherichia coli is one of the major bacteria of the natural intestinal microflora. One of the most important pathogenic strains of this species is Enterohemorrhagic *E. coli* (*EHEC*); *E. coli O157: H7*. The *E. coli O157: H7* infectious dose is low and only 100 of these bacteria are enough to cause the disease (3).

These bacteria have the ability to produce a toxic substance similar to the *Shigella* bacteria toxin, which is called

Shiga toxin (STX). They are classified in the group of bacteria producing hemorrhagic enterocolitis and Hemolytic uremic syndrome (4).

To control *E. coli O157: H7* pathogenesis, its colonization should be prevented. This bacterium must be able to compete with the intestinal bacterial flora to stick to the gut epithelium to get the nutrients it needs and ultimately cause infection in the host. Accordingly, the host intestinal bacterial flora capacity determines the presence and the amount of nutrients needed by the pathogen (5).

Histopathological studies indicate that *E. coli O157: H7* can infect many internal organs of the host body; therefore, prevention, rapid diagnosis and treatment of the infection caused by this pathogen are of great importance in controlling its pathogenicity and irreparable complications (6).

The choice of an appropriate animal model for investigating the intestinal probiotics' histopathologic effects against intestinal pathogenic bacteria is important. Its importance is from this view that the study of probiotic bacterial colonization in the intestine, the interaction between the microbe and the host, and also the tissue damage created in exposure to the pathogenic agent (through the creation of an oral infection) are possible with ease and the least cost and time in a live vertebrate host that follows a pathogenic pattern similar to that of humans. The studies have shown that zebrafish, known as *Danio rerio*, belonged to the *Cyprinidae* family, is an appropriate laboratory model for examining the pathogenesis of microbial pathogenic agents (7-11).

To the best of our knowledge, there has been no study on the effects of probiotic *L. acidophilus* in exposure to *E. coli O157: H7* pathogen in zebrafish. However, the probiotic role against other pathogens has been proven in this fish as a laboratory model (12).

2. Objectives

The aim of this research as a descriptive and qualitative study was to evaluate the effect of probiotic *L. acidophilus* on histopathological changes in the zebrafish intestine as a laboratory model before and after exposure to *E. coli O157: H7*.

3. Methods

3.1. Preparation of Probiotic

Lactobacillus acidophilus is a lactic acid bacteria and the strain used in this study was *L. acidophilus* La-5. The probiotic from the company Chr. Hansen of Denmark was prepared in the form of lyophilized (DVS Starter Culture) from the Nutrition and Food Research Institute of Iran at Shahid Beheshti University of Medical Sciences.

To prepare the required logarithm, initially, the primary lyophilized suspension of *L. acidophillus* was prepared. The bacterium was injected into the test tube containing MRS broth medium (Merck, Germany) and reached an appropriate concentration in low aerobic condition for 48 hours in CO_2 incubator at 35°C and 150 rpm. Then macroscopic and microscopic features of the bacterium were studied and biochemical identification was performed. In the next step, the growth opacity equal to 0.5 McFarland (1.5×10^8 CFU/mL) of bacterial suspension was prepared and stored at 4°C for further use in the sealed plastic vials with the same volume (13, 14).

3.2. Preparation of Pathogenic Agent

The *E. coli O157: H7* PTCC 12900 strain (with growth opacity equal to 0.5 McFarland), was prepared as lyophilized form from the Faculty of Veterinary Medicine, Tehran University of Medical Sciences and confirmed by microscopic examination and biochemical tests.

3.3. Experimental Design

This experiment was designed to investigate the effect of probiotic *L. acidophilus* on intestinal villi in zebrafish exposed to *E. coli O157: H7*. The test was carried out in 4 aquariums (tanks) on 48 pieces of zebrafish with an average weight of 0.6 gr. During the adaptation period, fish were fed with a commercial ration and no dietary supplement twice daily, based on 5% body weight. After the adaptation period, the light condition was adjusted according to the 12/12 hour cycle during the test period. The physicochemical parameters of water were also measured daily during this period. In this test, four groups of control (C1A1), treated with probiotic (T1A1), exposed to the pathogen (B1A1) and probiotic-treated exposed to the pathogen agent (T2A2) were considered in 2 replicates.

3.4. Preparation of the Ration

The probiotic suspension was prepared and mixed with 0.5 gr of commercial zebrafish (Tetra-Iran) feed and used for the treatment of T1A1 and T2A2 groups twice daily (15).

3.5. Exposure to the Pathogen

The exposure to the pathogen test was performed after 27 days of probiotic feeding (T2A2). The B1A1 and T2A2 groups were exposed to the PTCC 12900 strain of *E. coli O157: H7* (with growth opacity equal to 0.5 McFarland) with immersion method for 3 days. On the days 15 and 27, intestinal tissue samples were collected and cultured in MRS agar medium and colonization of probiotic bacteria in the intestinal tract of fish was compared with the control group (7, 16).

3.6. Histopathologic Evaluation

Intestinal tissue samples were taken for histopathologic studies on days 0, 15, 27, and 30 and fixed in 10% formalin. The samples were dehydrated with increasing percentages of ethanol solution. Each sample was then transferred to the xylene for 30 minutes. In the next step, the specimens were placed and fixed inside paraffin. The samples were cut by microtome into pieces of 5 μm thickness in transverse and longitudinal sections and quickly transferred to the slide, stained with H&E and stored at 40 - 50°C for 24 hours. The samples were evaluated by optical microscopy (Olympus BX51; Olympus, Tokyo, Japan) (17).

The histopathological changes including inflammatory response, necrosis and hemorrhage were investigated in the samples. In addition, in order to measure the length of the gut villi, the number of ten villi was randomly selected from the intestinal middle segment for evaluation (17).

The morphometric analysis was performed by the Image Proplus software version 6 (cyberneticsco, USA) and statistical analysis was done using repeated measures One-way ANOVA ($P < 0.05$, $P < 0.01$, $P < 0.001$).

4. Results

The findings showed that in the C1A1 group, in all regions, the intestine was normal with no histopathological changes. In B1A1 group, the natural structure of the intestinal tissue was preserved until day 27. However, on day 30 (3 days after the exposure to *E. coli O157: H7*), significant pathological changes were observed including severe epithelial cell death (Figure 1A and 1B), mild edema associated with the accumulation of inflammatory cells in lamina propria (gut mucosal membrane) (star), and severe necrosis (thick arrows) (Figure 2).

Studies also showed that the fish of the probiotic group (T1A1) had intestinal villi longer than the C1A1 and B1A1 groups; it was especially impressive on days 27 and 30 after treatment.

The number of goblet (mucosal) cells in this group significantly increased in comparison with the C1A1 and B1A1 groups on days 27 and 30 after treatment. The microscopic intestine images in the T2A2 group were closely similar to the T1A1 group until day 27.

On day 30 after treatment and after infection with *E. coli O157: H7*, the degree of necrosis variability of epithelial cells was evident in this group. However, this necrosis was significantly reduced compared to the B1A1 group. In addition, like T1A1, the number of goblet cells increased significantly in this group compared to the C1A1 and B1A1 groups (Figures 2 and 3).

5. Discussion

Recently, the use of microorganisms called probiotics for the prevention and treatment of digestive disorders has had a growing trend. Studies have shown that many probiotic species inhibit the growth and metabolic activity, adherence, and transport of the enteropathogenic bacteria to the intestinal cells (18-20).

The *E. coli O157: H7* serotype studied in this research is one of the major causes of enterohaemorrhagic (*EHEC*) digestive diseases (21-23).

The results of this study indicate a significant increase in the length of intestinal villi and reduction in the histopathologic effects caused by the *E. coli O157: H7* exposure, including degradation, edema, necrosis and death of epithelial cells, as well as increase in the goblet and mucosal cells that can be caused by the protective effect of the probiotic *L. acidophilus* consumption. The aim of this project was descriptive investigation of the effect of probiotics against the desired pathogen in reducing the tissue damage in the zebrafish intestine as a perfect and inexpensive model with ease of research. In this study, the cause and mechanism of the protective effect of probiotics have not been addressed.

In this regard, Pirarat et al., showed that probiotic supplementation was useful in improving the intestinal structure and its use increased the length of the villi significantly. Also, this study showed a significant increase in the population of mucosal cells such as goblet cells in all parts of the intestine in the probiotic group that is consistent with the results of the present study (17). Sherman et al. also found in a study that probiotics prevented the damage caused by *E. coli O157: H7* and *E. coli O127: H6* to the gut cells by decreasing the pathogen-induced effects and increasing the electrical resistance of the tight junctions of intestinal epithelial cells. This study was consistent with the present study in describing the usefulness of probiotics (24). Putaala et al. investigated intestine epithelial connections in histopathologic examination using four probiotic bacteria, including L. acidophilus NCFM, in exposure to E. coli O157: H7. The results indicated that the use of probiotic bacteria might be beneficial in the gut epithelial cells' preservation against harmful effects of the pathogenic bacteria, which was in line with the results of the current study (25).

In a study by Silva et al., with the aim of examining the effect of probiotic use in the ration on the intestinal tract of tilapia, the results indicated that probiotic consumption increased the length of the intestinal villi, especially in the upper part and also, the number of mucosal secretory cells (goblet cells). Although this project was different from the current project from the view of the experimental model and the type of probiotic bacteria used, the outcomes of

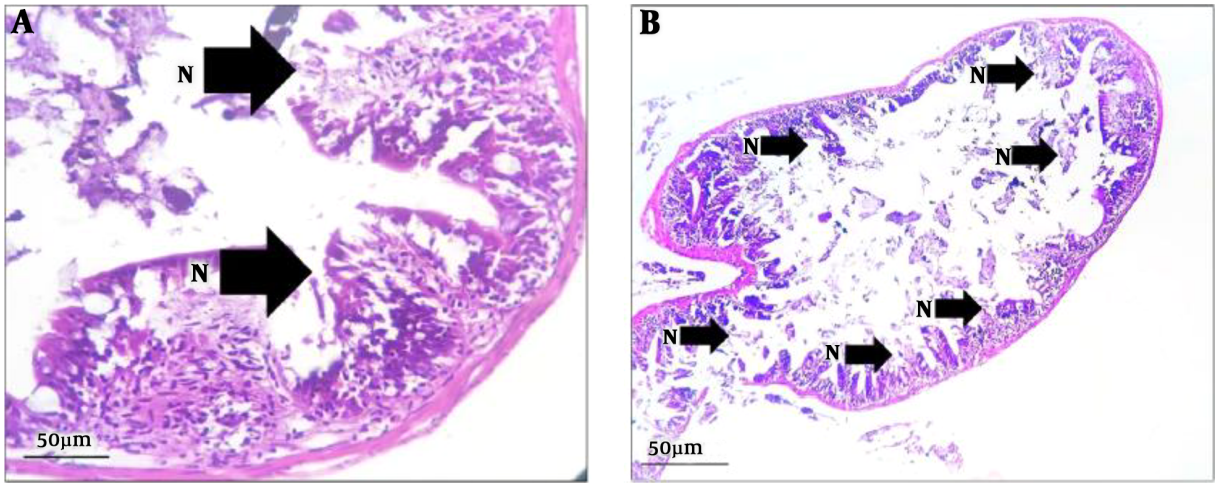

Figure 1. A: massive necrosis and pathological damage in zebrafish intestine after exposure to *E. coli O157:H7* in B1A1; N: necrosis; 400 X magnification; H&E staining. B: massive necrosis and pathological damage in zebrafish intestine after exposure to *E. coli O157:H7* in B1A1; N: necrosis; 100 X magnification; H&E staining.

the research are similar (26).

In consistence with the results of this study, Kim et al. showed that the use of *L. acidophilus* in the feed would increase the mucosal secretion and inhibit the binding of *E. coli O157: H7* to the epithelial cells, thereby, reduce the histopathological lesions resulting from it (27). In line with an increase in the knowledge in association with probiotic effects and potential mechanisms using the zebrafish model to evaluate the ability of this fish in *in vivo* condition; as well as the protective effects of probiotics on the intestinal mucosal immunity in response to the *Aeromonas hydrophila* infection, Wang et al. reported that lactic acid bacteria play a role in increasing the immunity level, stimulating the anti-inflammatory response and repairing the intestinal mucosa to protect zebrafish against infection. These researchers had given a special attention to the importance and protection of zebrafish as a powerful model for a better molecular understanding of the probiotic effects, which is confirmed in the present study as well (28).

A study on the effect of the probiotic *Bifidobacterium thermacidophilum* against *E. coli O157: H7* in mice reached prominent results of significant reduction in the intestinal damage and mucosal lymphatic reaction in a group fed with probiotic after exposure to the pathogen *E. coli O157: H7*. In this study, as with the results of the current study, despite the difference in the type of probiotic and host, the histopathological changes of the intestine were significantly reduced in the group treated with probiotic compared to the group which received the base ration alone (29).

Another study by Liu et al. compared the effects of two *Lactobacillus* strains, including *L. acidophilus*, on survival, growth, immunity and protection against the *Aeromonas hydrophila* infection in tilapia. The samples were collected from intestine, kidneys and spleen. The results showed no significant difference in survival, weight gain or nutrient conversion in different treatments, but nutrition protects fish against the effects of exposure to the pathogenic agent ($P < 0.05$). As a result, *L. acidophilus* was identified as an ideal criterion for choosing a food supplement in aquaculture (30).

In line with the positive effects of probiotic in zebrafish, Madsen et al. found that probiotics can create resistance against the disease by changing the microbial balance of the intestine, which is consistent with our study (31, 32). Medellin-Pena et al. studied the probiotic strain *L. acidophilus* against *E. coli O157: H7*, similar to the present study and in a different way in mice. The probiotic protective effect against this pathogen was proven through the molecules released by probiotics which affect the transcription of genes related to the colonization of the pathogen (2).

Kumar et al. also conducted an experiment on human gut cells in *in vitro* using five lactobacilli species and caused the infection by enteropathogenic *E. coli*. They showed that short-term treatment with *L. acidophilus* potentially inhibits the pathogen in the intestinal cells (32).

According to the present study, *L. acidophilus* can be used as a suitable probable candidate for nutrition in order to reduce the histopathological effects of *E. coli O157: H7* in aquatic intestine.

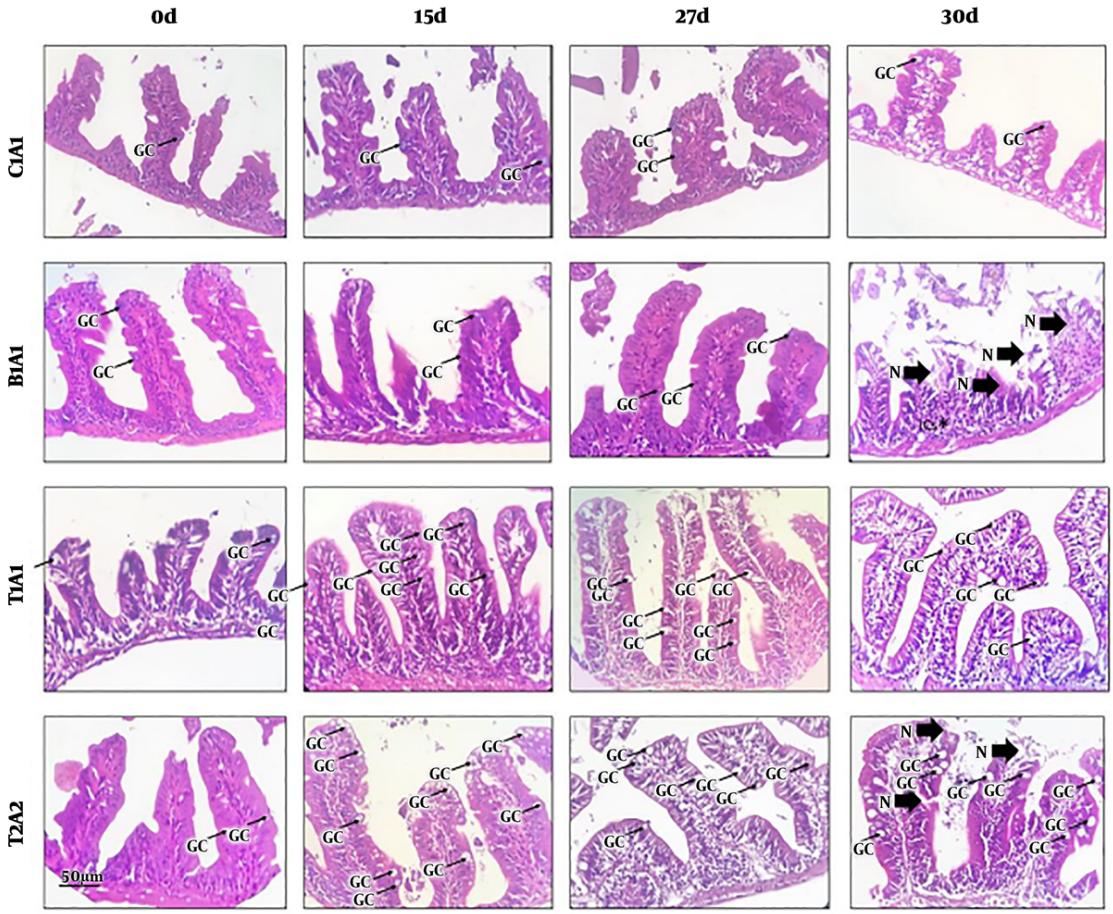

Figure 2. Histopathologic sections of harvested intestine samples in different experimental groups; GC, goblet cells; N, necrosis; ICs, inflammatory cells; 400X magnification; H&E staining.

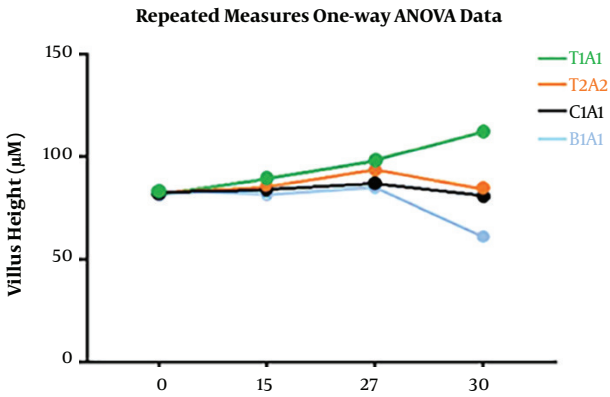

Figure 3. The height of villi in different experimental groups; day 15, $P < 0.05$: T1A1 vs. B1A1; day 27, $P < 0.05$: T1A1 vs. B1A1, T2A2 vs. B1A1, T1A1 vs. C1A1, T2A2 vs. C1A1; day 30, $P < 0.01$: T2A2 vs. B1A1, C1A1 vs. B1A1, T1A1 vs. C1A1, T1A1 vs. T2A2, $P < 0.001$: T1A1 vs. B1A1.

Footnotes

Authors' Contribution: Study concept and design: Reza Kazempoor and Reza Mirnejad; analysis and interpretation of data: Fattah Sotoodehnejadnematalahi; drafting of the manuscript: Seyedeh Shaghayegh Mirabdollah Elahi; critical revision of the manuscript for important intellectual content: Reza Kazempoor and Seyedeh Shaghayegh Mirabdollah Elahi.

Conflict of Interests: Nothing to declare.

Funding/Support: Mrs. Seyedeh Shaghayegh Mirabdollah Elahi's personal funding for dissertation.

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