



Evaluation of the Effect of Probiotics Isolated from Traditional Dairy Products on the Expression of *Stx1* and *Stx2* Genes of Verotoxigenic *Escherichia coli* (VTEC) in Laboratory Conditions

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Abstract

Background: *Lactobacilli* are commonly used organisms in the production of probiotics.

Objectives: The present study was conducted to examine the effect of probiotics isolated from dairy products on the expression of *Stx1* and *Stx2* genes in verotoxigenic *E. coli* (VTEC).

Methods: This experimental study was conducted during April and January 2016 in East Azerbaijan, Iran. At first, Hundred samples of traditional dairy products were collected. Then, using standard phenotypic and genotypic methods, the *Lactobacillus casei* (*L. casei*) and *lactobacillus* (*L. plantarum*) were studied. Subsequently, these strains were examined along with the pathogenic bacteria strain of *Escherichia coli* (*E. coli*) (O157: H7) by neighboring cultivation. After extraction of mRNA, the expression of these genes was determined using real-time PCR.

Results: The results revealed that *L. casei* reduces the expression of Shiga toxin (*Stx1* and *Stx2*) genes more compared to *L. plantarum* ($P = 0.001$). Moreover, it was found that both *Lactobacilli* reduce the expression of *Stx1* genes more than *Stx2*.

Conclusions: Probiotics could be used to prevent and control intestinal diseases caused by *E. coli*.

Keywords: *Escherichia coli*, Expression, Gene, Probiotic, Shiga toxin, *Stx1*, *Stx2*, Verotoxigenic

1. Background

Probiotics, including *Lactobacillus* species, are viable and beneficial microorganisms that can be used in humans or animals; they have an effect on the microbial flora of the body, leading to beneficial effects on the host health (1-4). Many studies have indicated that probiotics are important in preventing and curing intestinal disorders, such as diarrhea and pouchitis. Most probiotics belong to a large group of the microbial flora of humans' intestines and some of these microorganisms are strains of *Lactobacillus* bacteria that are rarely pathogenic to humans and animals (5). *Lactobacillus* is polymorphic, anaerobic, and nonmotile bacterium, and generates energy by fermentation of sugars (6, 7). The beneficial effects of probiotics on host protection, especially against digestive tract disease, are performed by inhibitor compounds such as organic acids (acetate), propionate butyrate, H₂O₂, and bacteriocin compounds. Among these compounds, bacteriocin with the most effective inhibitory activity against bac-

terial species has been most commonly studied (8). *Lactobacillus* commonly exists in yogurt and other dairy products that enhance the immune system, improve the intestinal absorption and digestion, eliminate pathogenic bacteria, prevent the binding of these agents to the intestinal wall, and compete with the pathogen (9, 10).

Among the available strains, at least 100 serotypes of *E. coli* are capable of producing Vero toxin. However, among these serotypes, *E. coli* O157: H7 in humans is known as EHEC. O157: H7 strain is one of the most important causes of intestinal infections (11). This bacterium produces severe bloody diarrhea and high-risk diseases, such as hemolytic uremic syndrome (HUS), hemorrhagic colitis (HC), and thrombotic thrombocytopenic purpura (TTP) (12, 13). This bacterium is known as one of the food-borne pathogens. Shiga toxin-producing *E. coli* (STEC) can survive for a long time in such foods as apples, sausages, and mayonnaise. One of the other abilities of this bacterium is the lower infectious dose (less than 100), and therefore all enterohemorrhagic *E. coli* strains observed in animals

are considered as risk factors for humans. However, the O157 serotype can cause disease in humans (14, 15). *E. coli* pathogenicity factors: H7 O157, such as toxins, adhesin, hemolysin, lipopolysaccharide, and flagellum play a significant role in low levels of infectious doses of this bacterium (16). Also, *E. coli* strains can disturb the integrity of cytoskeleton and cell-cell tight junction in host epithelial cells. Shiga toxin 1 & 2 (*Stx-1* and *Stx2*) are the most critical toxins produced by this bacterium (17). Shiga toxin and lipopolysaccharide are also the essential factors in the creation of HUS (13). It is also called Vero toxin because of the lethal effects of the toxin in the Vero cell line from African green monkey kidney (12). The consumption of antibiotics to treat and prevent infections caused by this bacterium not only produces drug resistance but also interferes with the normal digestive tract flora, making the body susceptible to a variety of intestinal diseases. On the other hand, the use of antibiotics in treatment causes the phage Stx carriers to pass the circulatory cycle (lytic) and increases the secretion of Stx and the progression of the disease to HUS in the antibiotics recipient (18-20). Thus, in this study, we determined the effect of *Lactobacilli* isolated from dairy products on the expression of *Stx1* and *Stx2* genes that involved in the pathogenesis of VTEC. Also, we conducted this study taking into account the following factors: the importance of this bacterium in acute illness; low infectious doses; and the presence of various pathogenic agents, such as the production of toxins and their transfer by food. In conducting this study, we also considered that previous studies have indicated that probiotics play a significant role in the prevention of intestinal diseases.

2. Methods

2.1. Sample Collection

This experimental study was performed in East Azerbaijan province, Iran, from during April 2016 to January 2016. Samples were collected from local dairy products (yogurt and cheese) in the villages around city of Ahar in East Azerbaijan province, Iran. With the population of 3.725 million, East Azerbaijan province is diverse in climate, and its economy depends on large industries and agricultural and commercial activities. The number of examined samples was large enough, so that at least one isolate could be studied from each of the probiotics.

2.2. Cell Cultures

Sampling was based on Morgan formula, which was calculated to be 100 samples. Samples of dairy products were collected in sterile conditions and transferred in sterile containers (Falcon 50 ml) to the laboratory in the vicinity of ice. First, we ensured that equipment was calibrated.

Then, bacterial suspension was prepared by Kalycha and its associated method using PPS solution and primary culture of bacteria in MRS liquid culture medium (Micro media). Next, bacterial cultures were transferred to the PBS medium with pH 2.5 (pH meter -Met Rohm-Sweden) to be isolated based on Erkila Pnajah method; isolates were resistant to Acid. After determining the survival percentage of isolates in the acidic medium, the resistance of the isolates to bile salts was determined using Gil Liland et al. method in a liquid MRS medium with 0.3% Oxall (Merck). Spectrophotometric apparatus every half an hour recorded growth in control and treatment cultures by measuring the absorbance at 600 nm wavelength using spectrophotometer (Pharmacia-Biotech-England). The absorption curve was plotted based on incubation time. After identifying the probiotic potential of isolated strains based on the proposed method in the Barji and Asnes book in 1986 and Wood and Holzappel in 1995, the phenotypic isolates were identified using catalase test, gram coloring, growth at temperatures, and different pH, and tolerance to the concentration of different salts.

2.3. Study of Gene Expression

The next step was to identify acid-base and Beyle resistant strains using SrRNA molecular methods and sequencing. PCR reaction was performed using specific primers, such that DNA was extracted from all isolates using the C-TAB method reported in the sources or using the DNA extraction DNPTM kit (High yield DNA purification- Sina-colon, Iran). One percent agarose (Invitrogen-USA) gel for electrophoresis was used to evaluate the qualification of the extracted DNA. Specific primers for the propagation of 16SrRNA fragments for *Lactobacillus* strains were designed using oligo7 software and blast with 16S rDNA sequences found on the NCBI GeneBank site.

After selecting probiotic potential strains, the antibacterial effect of these strains on the O157H7 bacilli were performed using a disc diffusion method with a 24-hour filtered medium of selected *Lactobacilli*. Bacteria with antibacterial effects cannot be used to study the effects on the expression of the enterotoxin gene of *E. coli*, as these bacteria will be extinct after exposure to bacteriocins, and gene expression will stop. However, one example of bacteriocin *Lactobacilli* can be used as a negative control. Finally, the effect of four bacteria of *Lactobacillus* of different species with probiotic potential and two standard strains of plantarum and casei, which are available in the market as probiotic pills, were investigated using neighboring cultures along with *coli* O157 strain bacteria. Inclusion criteria were based on *Lactobacillus* phenotypic characteristics and resistance to bile salts. The bacteria that were not resistance

to bile salts and did not have *Lactobacillus* - related genotypic and phenotypic characteristics were excluded from this study. The presence of *Stx1* and *Stx2* genes for the mentioned strains was confirmed using PCR. Neighboring cultures of O157: H7 and O57: H7 VTEC with probiotic bacteria was done in the near term after 1, 4, and 8 hours. Then, RNA was extracted from adjacent bacteria using RNA extraction kit (Mini-Preps Kit -Canada), according to the protocol of the company. The quantitative and quantitative information of the extracted RNA was evaluated by nanodrop instrument (NanoDrop ND-1000 spectrophotometer). Initial inoculation of both bacteria and probiotics with opacity was OD = 0.5, both bacteria were in the logarithmic phase of growth. After extracting RNA using synthetic kit of cDNA, cDNA was prepared from RNA. About 1 μ g of RNA was converted to cDNA by the Rovers transcript enzyme of the MMLV (Takara, Japan), at the temperature of 42°C for one hour using thermocycler (Takara -Japan). The obtained cDNA was kept in the refrigerator-70 (Bosch-Germany) until quantitative PCR was performed. For real-time PCR experiments, primer was designed for *Stx1* and *Stx2* genes and sent to a German company (MWG) or another company for synthesis. In addition, by designing a primer pair for the 16srRNA gene, this gene was used as a control, as a data normalizer in quantitative PCR, and as a housekeeping gene. Quantitative PCR was done by Cyber-Greens method and QuantiTect SYBR Green PCR kit. Four local probiotic bacteria with two standard probiotic bacterial isolates from probiotic pills in the medicine market (six probiotic bacteria and two strains of pathogenic bacteria) were used as positive control and in triplet. The standard diagram was drawn using five - times cDNA dilutions of a positive affinity with factor 10. Specificity control of reproduction was performed by plotting the melting curve. The obtained data were analyzed using the Delta-Delta-Ct formula ($\Delta\Delta CT$) proposed by pfaffl in 2001 and by Rest program.

2.4. Statistical Methods

Data were analyzed using SPSS software, Version 20 (IBM Corp., Armonk, N.Y., USA). The results that showed a normal distribution were compared using one-way ANOVA, and the results that were not normally distributed were compared by Mann-Whitney U non-parametric test ($P < 0.05$).

3. Results

Isolates of the strains of *L. casei* and *plantarum* of dairy products, such as yogurt and cheese, were randomly selected from the villages around city of Ahar, Iran. It was

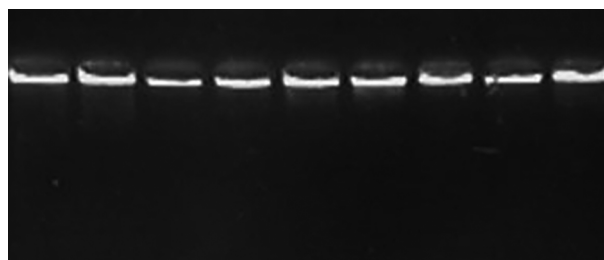


Figure 1. Electrophoresis of DNA extraction from isolated strains

assured that there were no commercial strains and sterile conditions in this area. Then, using standard phenotypic methods, such as isolating acid resistant strains and determining their resistance to acid and determining the tolerance of isolates to bile salts, *lactobacilli* phenotypes were identified.

3.1. DNA Extraction

After extraction of DNA from isolated bacteria, about 5 μ L of extracted DNA in 0.8% agarose gel was loaded. After performing electrophoresis for one hour at a voltage of 100 volts, the quality of the bands was studied under UV rays. Then, samples of DNAs with good bands quality were selected. Figure 1 demonstrates the DNA extraction sample.

3.2. Reproduction of *Lactobacillus* Isolates of 16s rRNA Sequences

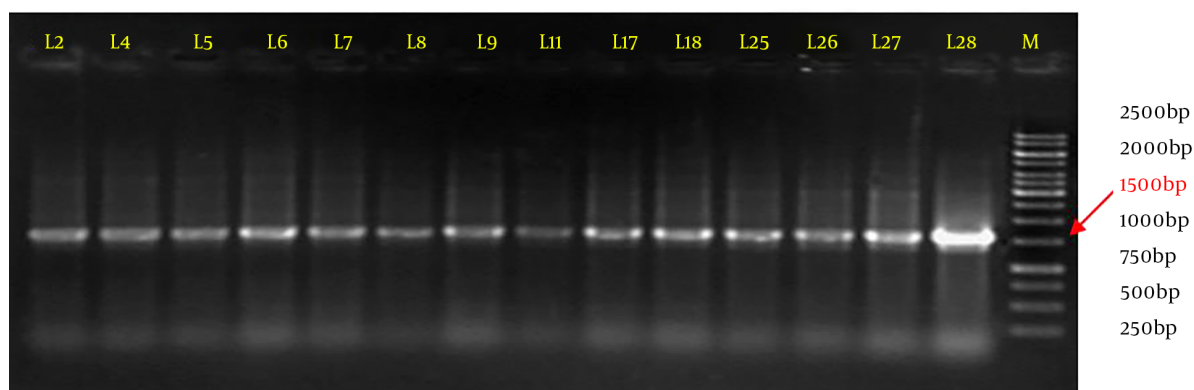
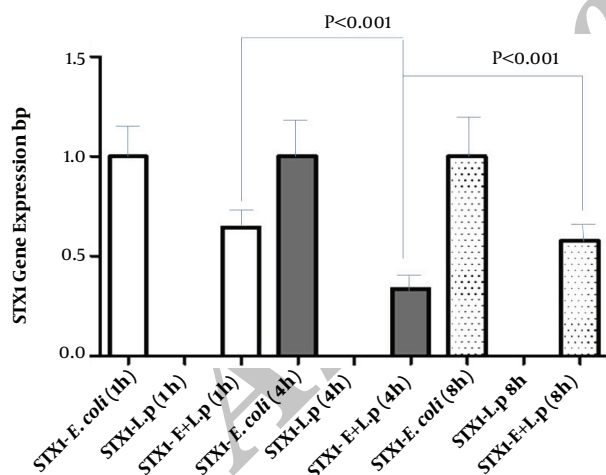
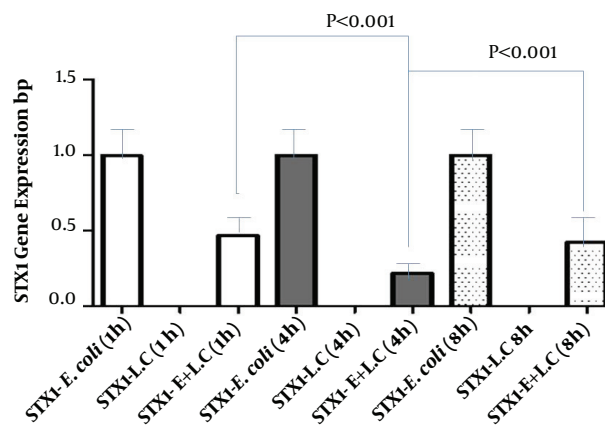
Sequences of 16 s rRNA isolates were amplified using a pair of primers designed during polymerase chain reaction (Figure 2).

As seen in the proliferation proliferation products were obtained at approximately 1500 pairs of nucleotides and as a single strip. The 1- kb DNA Ladder was used to represent the size of the plots. The electrophoresis of the amplified products in agarose gel was 1.5%.

In the present study, *Stx1* and *Stx2* specific primers were used as internal controls using SYBR GREEN and 16S rDNA genes. In Muller Hinton medium, the bacteria of *L. caesi* and *plantarum* with *E. coli* (from each bacterium with OD = 0.5 cc) were incubated for 1, 4, and 8 hours at 37°C. After centrifugation of the bacteria, RNA was extracted using thrysol. After nanodrop and determining the amount of RNA, cDNA synthesis was performed. Results revealed that *lactobacilli caesi* and *plantarum* affect the expression of Shigotoxin and can affect the virulence of the *E. coli* bacterium and decrease its growth. In addition, results showed that *L. caesi* reduced the amount of gene expression in comparison to *plantarum*. The results of culture revealed that in four hours cultivation, the gene expression

Table 1. The Sequence of the Primers

Size, bp	Sequence	Primer	Gene
555	TTCGCTCTGCAATAGGTA	Stx1-F	Stx-1
	TTCCCAGTTCAATGTAAGAT	Stx1-R	
118	GTGCCTGTTACTGGGTTTTCTTC	Stx2-F	Stx-2
	AGGGGTCGATATCTCTGTCC	Stx2-R	

**Figure 2.** Electrophoresis of proliferation products of 16 s rRNA sequences for *Lactobacillus* isolates strains**Figure 3.** Effect of *L. plantarum* on the expression of *Stx1* gene in real-time PCR results (results were reported as a median based on non-parametric and one-way ANOVA tests using SPSS, Version 20.)**Figure 4.** Effect of *L. casei* on the expression of *Stx1* gene in real-time PCR results (results were reported as a median based on non-parametric and one-way ANOVA tests using SPSS, Version 20)

in both bacteria decreased more than 8 hours. It is likely that as the time passes, the effects of *L. casei* and *plantarum* on the *E. coli* are reduced. Also, the results indicated that both *lactobacilli* reduced the expression of *Stx1* gene more than *Stx2*, and there was not a significant difference between them ($P < 0.001$).

4. Discussion

This study showed that cheese and yogurt produced by traditional methods are an important source for probiotic bacteria. After isolation and identification of *casei* and *plantarum lactobacilli*, the effect of these bacteria on the expression of *Stx1* and *Stx2* genes was studied. Many studies have been conducted on probiotic species of *Lac-*

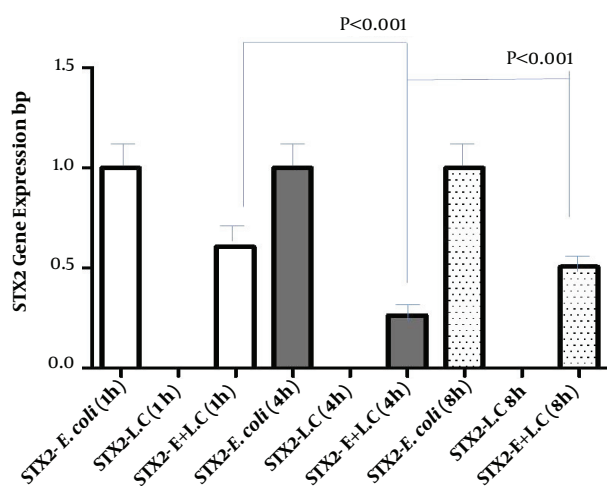


Figure 5. Effect of *L. casei* on the expression of *Stx2* gene in real-time PCR results (results were reported as a median based on non-parametric and one-way ANOVA tests using SPSS, Version 20)

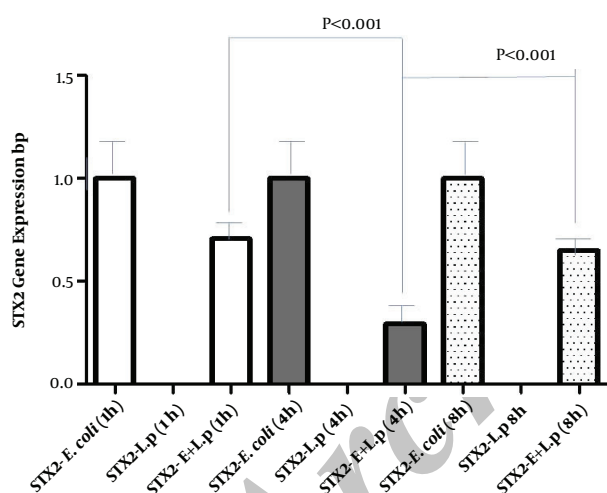


Figure 6. Effect of *L. plantarum* on the expression of *Stx2* gene in real-time PCR results (results were reported as a median based on non-parametric and one-way ANOVA tests using SPSS, Version 20)

tobacillus and their effects on pathogenic bacteria, such as *E. coli* in dairy products; and they have indicated that these bacteria can reduce the growth of *E. coli*. However, to date, no studies have been conducted on *casei* and *plantarum* species. The present study found that *L. casei* reduces the amount of gene expression more than *plantarum*. The results of culture showed that in 4 hours cultivation, the gene expression in both bacteria decreased more than 8 hours. It is likely that with the passage of time, the effects of *L. casei* and *plantarum* on the *E. coli* are reduced. The results also indicated that both *Lactobacilli* reduced the

expression of *Stx1* gene more than *Stx2*. Silva et al. (21) demonstrated that *L. rhamnosus* GG has anti-bactericidal effects against a variety of pathogens strains, such as *Pseudomonas*, *Salmonella*, *Clostridium*, and *E. coli* B-44. Also, other studies showed that *L. plantarum* exerted a beneficial effect against the damage to integrity of Caco-2 mono-layer cells, which was previously infected by Enteroinvasive *Escherichia coli* (EIEC) infection (22). However, it can be concluded that *L. casei* is superior to the plantarum strain on the growth of the *E. coli* bacteria. In this study, due to the importance of Enterohemorrhagic *E. coli* (EHEC) pathogenesis strains O157: H7, the inhibitory effect of *Lactobacillus* on the expression of *Stx-2* and *Stx-1* gene was evaluated. By examining the effect of *Escherichia coli* O157: H7 strains with different genes in laboratory and intracellular conditions, many scientists have shown that strains of *Stx-2* gene compared to *Stx-1* gene strains or both *Stx-1* and *Stx-2* genes are more pathogenic. Their finding was also confirmed by our results. Ogunbanwo et al. investigated antimicrobial activity and the production of bacteriosins by *Lactobacillus* in 2003. The researchers, using the wells method, showed that *Lactobacillus* inhibits the growth of *E. coli*, *Bacillus cereus* (*B. cereus*), and *Yersinia enterocolitica* (23). Wolf et al. in 2000, using the wells method showed that the fermented milk mixture containing *L. casei* inhibited the growth of intestinal pathogens including *Shigella dysentery*, *Salmonella typhimurium* (*S. typhimurium*), and *E. coli* (24). With respect to the antibacterial effect, *Lactobacillus* produces non-bacteriocidal substances that are unknown and different from lactic acid. Also, it extensively inhibits the growth of Gram-positive and negative pathogens, such as *Staphylococcus aureus* (*S. aureus*), *Listeria monocytogenes* (*L. monocytogenes*), (*S. typhimurium*), *Shigella Flexneri* (*S. Flexneri*), *Klebsiella pneumonia*, *Pseudomonas aeruginosa* (*P. aeruginosa*), and *Bacteria*. In 2003, in a laboratory experiment, Lee et al. found that *L. casei* strain of Shirota, in competition with gastrointestinal bacteria, prevented binding to the Caco-2 cell line about 46%. In this study, it was determined that the highest inhibition of *L. casei* Shirota (Above 30%) is on *E. coli* TGI (*S. typhimurium*), E10, *E. coli* ATCC1775, and (*S. typhimurium*) ATCC14028 (25). In 2003, Laura introduced the mechanism of inhibitory effect of *Lactobacillus* and *Bifidobacterium* on pathogenic bacteria by reducing the pH of the intestinal environment. This was done by producing short chain volatile fatty acids, the use of nutrients, and the production of certain antimicrobial compounds, such as bacteriosins (26). In 2000, Saarela reported that *Lactobacillus acidophilus* (*L. acidophilus*) intercropping reduces the life of *S. aureus*, *S. Flexneri*, *E. coli*, *Klebsiella pneumonia*, *B. cereus*, *P. aeruginosa*, and *Enterobacteriaceae* strains in culture media. Antimicrobials with low molecular weight, which are

independent of lactic acid production, have no effect on *Lactobacillus* or *Bifidobacterium* strains. The antimicrobial activity of this bacterium against *Salmonella typhimurium* (*S. typhimurium*) has also been shown in infected mice (27, 28). Conconnier et al. reported that the use of *L. casei*, *L. acidophilus*, and *Lactobacillus lactis* (*L. lactis*) supernatant culture has a bactericidal effect on the wide range of gram-positive and gram-negative pathogens. In the above studies, the highest inhibitory effect of *L. plantarum* is clear in the results on all three pathogens of *Shigella dysentery*, *S. typhimurium*, and *E. coli* (29). It has been shown that EHEC O157 produces a autoinducer-2 (AI-2) and it is used for bacterial interspecies relationships (30, 31). This molecule was first defined as a furanosyl borate diester (32); and it was later proved that the enzyme responsible for AI-2 synthesis was encoded by the *luxS* gene in EHEC O157 (33). In their 2007 study, Medellin et al. indicated that *L. acidophilus* has strategies that interfere with QS regulation of pathogens in foods such as *E. coli* O157, indicating that *L. acidophilus* reduces the production of AI-2 molecules by *E. coli* O157 (34); the reported results confirm the results of our study. In our study, native *Lactobacillus* was assessed using molecular technique and in the genetic level, unlike other studies that assessed the phenotypic view, and this was strong point of present study. It is recommended that other bacterial pathogenic agents involved in pathogenesis be studied in future studies.

4.1. Conclusion

L. casei and *L. plantarum* isolated from dairy products can reduce the expression of *Stx1* and *Stx2* genes, which are a component of bacterial pathogenicity. Also, these two genes can be useful in controlling the infection caused by this bacterium.

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Footnotes

Conflict of Interest: The authors declare that they have no conflict of interest.

Ethics Statement: The article did not conduct any studies on patients. Moreover, this article did not contain studies with human or animal subjects performed by any of the authors that needed to be approved by the ethics committee.

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