Antagonistic Activity of Probiotic Bacteria Isolated from Traditional Dairy Products against *E. coli* O157:H7

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**Abstract**

**Background:** Probiotics are living microorganisms that have useful effects on health of digestive system when acquired in a defined dosage. *E. coli* O157:H7 is known as one of the most important agents of diarrhea in developing countries. Therefore, attention to the treatment of such gastrointestinal disease is essential. The aim of this study was to determine antagonistic activity of food born lactobacilli against *E. coli* O157:H7.

**Methods:** Lactobacilli were isolated from traditional dairy products (yogurt and buttermilk samples). Then, they were characterized using biochemical and molecular tests. *Bifidobacterium bifidum* PTCC 1644 was obtained from the microbial collection of Iranian Research Organization for Science and Technology in Lyophilized form. Similarly, *E. coli* O157:H7 PTCC12900 was obtained from faculty of veterinary medicine university of Tehran. The antagonistic activity of probiotics supernatants against *E. coli* O157:H7 was investigated using the disk diffusion agar, well diffusion agar and pour plate methods.

**Results:** The isolates were characterized as *Lactobacillus plantarum* and *Lactobacillus fermentum*. All isolates showed antagonistic activities against *E. coli* O157:H7 in all of the three methods, where the activity of *L. plantarum* and *B. bifidum* PTCC 1644 was greater than that of *L. fermentum*.

**Conclusion:** Metabolites produced by the probiotic bacteria are able to inhibit the growth of *E. coli* O157:H7. This can be an important solution for the prevention and treatment of *E. coli* O157:H7 infection and ultimately improve human health.

Introduction

_Lactobacillus_ and _Bifidobacterium_ spp., the main genera of lactic acid bacteria (LAB), are the most commonly used probiotics (1). It should be noted that all of the lactic acid bacteria are not necessarily probiotic. The microorganisms known as probiotic are added to food products, when these foods are eaten, the microorganisms enter the bowel and exert their effect there. The trend of consuming probiotic foods has gradually increased in Europe, Asia, and North America, and today these food products are offered in the majority of supermarkets around the world (1, 2). The mechanism of action of probiotic bacteria on improve of health can be attributed to the following points: settlement and intestinal proliferation, regulation and control of microbial flora of the intestine, prevention from adhesion of pathogenic bacteria to the intestine's mucus and deactivating them, prevention and treatment of digestive disorders, activity against pathogenic agents, regulation of the enzymatic activity of bacteria, prevention from side effects of treatment with antibiotics, alteration of the proteins in diet, primary digestion of proteins, synthesis of vitamins, improvement of calcium absorption, metabolism of lactose and reduction of lactose intolerance, improvement of digestibility, enhancement of the nutritional value of food products, prevention from allergies, reduction of blood cholesterol, enhancing the power of the body's immune system, anticancer effects, and reduction of the growth of non-useful bacteria (3, 4, 5). This group of bacteria and their produced metabolites can have a wide range of therapeutic uses. The studies confirm the positive role of these bacteria in inhibition of pathogenic agents. _E. coli_ O157:H7 is one the most important agents in the development of intestinal infections in human. These bacteria create a severe bloody diarrhea and some high-risk diseases such as hemolytic uremic syndrome (HUS), hemorrhagic colitis, and thrombotic thrombocytopenic purpura (TTP). _E. coli_ O157:H7 has also been known as one of the pathogens that transfer from food and is one of the natural intestinal flora of animals including cow, goat, and sheep. Accordingly, contaminated dairy products such as non-pasteurized milk, meat products, and undone hamburger are other important ways of transmission of this bacteria (6, 7). Nowadays, with the increase in antibiotic resistance and the side effects caused by of chemical drugs, application of alternative therapies seems to be necessary. The aim of this study was to evaluate the Antagonistic activity of probiotic bacteria isolated from Traditional dairy products against _E. coli_ O157:H7.

Materials and Methods

_Bacterial Strains and Isolation_

For Identification of _Lactobacillus_ spp. 9 dilutions of traditional yogurt and buttermilk (Collected in winter 2016 from Guilan province, Northern Iran) were prepared. From the three last dilutions, 2 ml was transferred to MRS Broth medium and incubated for 24-48 h at 37°C under anaerobic conditions. Then, to isolate the bacteria from the Broth medium, first the top solution of the culture was centrifuged at 3000 rpm for 10 min, where the precipitates in the bottom of the tube, a full loop was cultured on to the MRS Agar culture medium, then incubated at 37°C for 48-72 h under anaerobic conditions. The isolated colonies were identified by using biochemical tests, fermentation of carbohydrates including lactose, rhamnose, galactose, maltose, mannose and sorbitol along with microscopic and molecular studies. _B. bifidum_ PTCC 1644 was obtained from the microbial collection of Iranian Research Organization for Science and Technology in Lyophilized form where the _E. coli_ O157:H7 PTCC12900 was obtained from faculty of veterinary medicine university of Tehran with turbidity equivalent to 0.5 McFarland (Table 1).

_Molecular identification_

The second stage of identification of _Lactobacillus_ spp. was carried out using molecular
methods. For DNA extraction from the mentioned isolates, lysozyme enzyme was used. First, 1 ml of the culture medium containing the bacteria from the 24h cultivation of the lactobacilli was poured into a 1.5 ml microtube, and centrifuged at 3500 rpm for 8 min. Following the centrifuge, the top phase was removed and the precipitates were washed three times with the TEN (NaCl 150 mm, EDTA 100 mm, Tris) buffer, each time at 3000 rpm for 5 min. A buffer composed of 200 μl TEN buffer and 10 μl of lysozyme enzyme was added to the resulting precipitates, and they were incubated at 37°C for 30 min. following the passage of this time, 75 μl SDS was added and after sampling, incubated at 75°C for 30 min. In the next stage, 250 μl equilibrated phenol was added to the solution and then incubated at 52°C for 30 min. Next, 250 μl of chloroform was added to the solution and mixed well. Thereafter, the solution of interest was centrifuged at 12000 rpm for 15 min. Next, the aqueous phase was transferred to a new vial, with chloroform being added at a volume the same as the dragged aqueous solution. Following several times of vigorous shaking of the solutions, the vials were placed inside a centrifuge for 15 min at 12000 rpm, where the top phase was transferred to a new vial. Cold Isopropanol was added with a volume equal to the volume of the top phase and the vials were centrifuged at 13000 rpm for 15 min at. The top phase was then fully removed, and 400 μl cold ethanol was added to the resulting precipitates. In the next stage, the vials were exposed to room temperature for 15 min and then placed inside an open-air incubator at 42°C for 15 min, so that the alcohol would evaporate. Finally, the precipitates were dried at room temperature. Polymerase Chain Reaction (PCR) was employed to amplify bacterial 16S rRNA gene. The master mix for the PCR was prepared as follow: 3μL of 10× PCR buffer, 1μL of 25mM MgCl$_2$, 3μLof 10mM dNTP mix, 0.5μL of Taq DNA Polymerase, 12.5μL of MilliQ water and 1μL of each of the forward and reverse primers. Finally, 3μL of each DNA template was added in the corresponding tubes to make up the final reaction volume of 25μL. After PCR the product of PCR was electrophoresed in a 2% (w/v) agarose gel. The 1Kb ladder was used as a guide of the sizes of the bands. The thermal cycler was programmed as follows: 5 min at 94°C; 30 cycles of 45s at 94°C, 1 min at 58°C, 1 min at 72°C and 10 min at 72°C (11). Following DNA extraction, in order to determine the sequence, the PCR products sent to Macrogen Inc. (Korea). After sequencing, the sequence of the intended samples was viewed using Chromas software, and subsequently sequence analyses conducted in the blast program, NCBI.

**Preparing supernatant of probiotic bacteria**

Probiotic bacteria were incubated in MRS medium and anaerobic conditions at 37°C for 24 h with turbidity equivalent to 0.5 McFarland (1.5× 10$^8$ CFU/ml). Bacterial cells were removed by centrifuging the culture at 12000 g for 15 min.

**Antagonistic assay**

Disc diffusion agar, well diffusion agar and Pour plate method used to detect antagonistic activities of supernatants produced from probiotic bacteria. Mueller Hinton agar was used to measure the inhibitory effect and repeated three times to reduce error.

**Well Diffusion Agar**

In this method, culture was done on Mueller Hinton agar (MHA) medium with a sterile swab from a suspension of *E. coli* O157:H7 in TSB Broth medium (0.5 McFarland). Wells were created using a sterile Pasteur pipette on the medium and 100 μl of supernatants of probiotic bacteria were added into each well. The culture plates were incubated at 37°C for 24 h and the zones of inhibition measured in diameter (mm).

**Disk Diffusion Agar**

In this method, sterile paper discs (6 mm-Himedia) was treated in the supernatants probiotic bacteria with different concentrations of 100, 150,
and 250 μl. From suspension of *E. coli* O157:H7 cultured in TSB Broth medium (0.5 McFarland), culture on MHA was done using a sterile swab, and then the impregnated disks were placed on the surface of MHA with a certain distance from each other and the edge of the plate. The plates were then incubated for 24 h at 37°C. After incubation antagonistic activity was measured in diameter (mm) around the paper discs.

**Pour plate method**

Using six tubes, dilutions were prepared out of the milk sample. In the first tube, 1 ml of milk was poured, while in all the tubes, 9 ml distilled water was poured. Next, 1 ml of the main tube was inoculated to the other tubes. As little as 1 ml of the probiotic bacteria suspension and *E. coli* O157:H7 with a turbidity of 0.5 McFarland was inoculated to tubes 4 and 5, and after 4, 12, and 24 h, the contents of the tubes were cultured on MRS Agar, MacConkey Agar, and *Bifidobacterium* Agar. Following incubation at 37°C, the grown colonies of lactobacilli and *E. coli* O157:H7 were counted individually. Antagonistic tests were done in thrice and the mean values were recorded.

**Results**

*L. plantarum* and *L. fermentum* were detected by biochemical test. Also in molecular method after completion of electrophoresis, isolated bacterial had a band about 1465 bp. The PCR product was sequenced for 16S rRNA (Macrogen, South Korea) and the sequence was submitted to GenBank (NCBI) website (http://www.ncbi.nlm.nih.gov/BLAST.html) and blasted with other published sequences from the GenBank database. According to sequencing results the isolated bacterial identified as *L. plantarum* and *L. fermentum* (Figure 1). In all of the three methods of investigation, the supernatant of probiotic bacteria indicated antimicrobial activities (Table 2), where this activity varied between 4 - 8 mm in the Well and disc methods. *L. fermentum* had the lowest activity when compared with the two other bacteria (*L. plantarum* and *B. bifidum*). With the increase in the dilution in the disk method, antimicrobial activity increased. In the pour plate method, both *Lactobacillus* and *B. bifidum* indicated the greatest inhibition effect within 24 h.

**Discussion**

Lactobacilli and bifidobacteria, known as starters for production of dairy products, are the main agent for fermentation and protection of food. These bacteria also play major roles in development of smell, taste, and texture of fermentation products. Further, by preventing intestinal infections, decreasing cholesterol, stimulating immune system, and decreasing the risk of colon cancer, they play a substantial role in human health. By producing lactic acid and organic acids, these bacteria cause decreased pH of the environment, inhibiting the growth of many bacteria. Due to production of antimicrobial compounds including bacteriocin, these bacteria can be used as natural preservatives (8, 23). The antimicrobial properties of probiotic bacteria have been proven by many researchers (9,10,11,12,13,14,15, 24, 25, 26).

Enterohemorrhagic *E. coli* O157:H7, is one of the main human pathogens, found in contaminated food and water. Development of infection by this bacterium in human manifests itself as diarrhea without bleeding and in severe cases as severe intestinal inflammation along with bleeding (16). Inhibition of this bacteria through probiotic bacteria is of crucial importance. Ota et al., reported that consumption of yogurt causes more lactobacilli to be colonized in the intestine, thereby providing some conditions to prevent colonization of enterohemorrhagic *E. coli* (17).
Table 1. Identification of *Lactobacillus* spp. based on biochemical and morphological tests.

<table>
<thead>
<tr>
<th>Probiotics bacteria</th>
<th>Catalase</th>
<th>Motility</th>
<th>Fermentation of Carbohydrates</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Galactose</td>
</tr>
<tr>
<td><em>L. fermentum</em></td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>L. plantarum</em></td>
<td>-</td>
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</tbody>
</table>

Table 2. Antagonistic activity of isolates probiotic bacteria.

<table>
<thead>
<tr>
<th>Probiotics bacteria</th>
<th>Well diffusion agar (mm)</th>
<th>(mm) Disk diffusion agar</th>
<th>Pour plate method in period of time CFU × 10⁵</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>4h</td>
</tr>
<tr>
<td><em>L. fermentum</em></td>
<td>5</td>
<td>5</td>
<td>7</td>
</tr>
<tr>
<td><em>L. plantarum</em></td>
<td>8</td>
<td>7</td>
<td>7</td>
</tr>
<tr>
<td><em>B. bifidum</em></td>
<td>8</td>
<td>7</td>
<td>7</td>
</tr>
</tbody>
</table>
Zhao et al., also showed that probiotic bacteria are able to decrease *E. coli* O157:H7 in animals that transmit this bacteria (18). In the investigation by Gagnon et al., the effect of 5 strains of *Bifidobacterium* isolated from human origin was compared with *E. coli* O157:H7, where by the two isolates of *B. bifidum* RBL 71 and *B. bifidum* RBL B. 460 were able to indicate a good inhibitory effect (19). In the study by Brashears et al., 686 colonies isolated from goat stool were investigated for identification of lactobacilli. Following identification of the tolerability of acidic and alkaline conditions, 15 genera had the ability to inhibit *E. coli*, where *Lactobacillus crispatus* was the most competent strain (20). In this study, lactobacilli and *Bifidobacterium* strains also had an inhibitory effect against *E. coli* O157:H7, with the average 4-8 mm. Matsusaki et al., investigated the inhibitory effect of lactobacilli on the growth of pathogenic bacteria in concurrent culture (21), and as with this study, it was found that the growth of pathogenic bacteria diminished when exposed to these bacteria. Mohammaddoost et al., comparing well and disc methods to measure the inhibitory effect of lactic acid bacteria against pathogenic bacteria, it was found that well plate method was far more sensitive than the disk method (22). In the study by Shu et al., *Bifidobacterium lactis* HN019 can reduce of infection *E. coli* O157:H7, and suggest that this reduction may be associated with enhanced immune protection conferred by the probiotic (27).

**Conclusion**

In this study, the metabolites produced by the investigated bacteria were able to inhibit the growth of *E. coli* O157:H7, which can be an important solution in the prevention and treatment of *E. coli* O157:H7 infection and improve human health.

**Acknowledgements**

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**Conflict of interest**

No conflict of interest associated with this work.

**References**


