J Med Bacteriol. *Vol. 6, No. 3, 4 (2017): pp.23-30* *jmb.tums.ac.ir*

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

Maryam Rahimpour Hesari [1], Reza Kazemi Darsanaki [2], Ali Salehzadeh [1]*

[1] *Department of Biology, Rasht Branch, Islamic Azad University, Rasht, Iran.*

[2] *Young Researchers and Elites Club, Lahijan Branch, Islamic Azad University, Lahijan, Iran.*

ARTICLE INFO

Article type:
Original Article

Article history:
Received: 19 Jan 2017
Revised: 03 Mar 2017
Accepted: 11 Sep 2017
Published: 15 Oct 2017

Keywords:
Antagonistic activity, E. coli O157:H7, Probiotic bacteria, PCR, Traditional dairy products.

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. bidifum* 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.

• ***Please cite this paper as:*** Rahimpour Hesari M, Kazemi Darsanaki R, Salehzadeh A. Antagonistic Activity of Probiotic Bacteria Isolated from Traditional Dairy Products against *E. coli* O157:H7. *J Med Bacteriol.* 2017; **6** (3, 4): pp.23-30.

*Corresponding Author: Ali Salehzadeh, Department of Biology, Rasht Branch, Islamic Azad University, Rasht, Iran.

Tel: +98- 013-334-24094, *E-mail:* salehzadehmb@yahoo.com, salehzadeh@iaurasht.ac.ir

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 94ºC; 30 cycles of 45s at 94ºC, 1 min at 58ºC, 1 min at 72ºC and 10 min at 72ºC (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 Gen Bank (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. fermantum* (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.

Probiotics bacteria	Catalase	Motility	Fermentation of Carbohydrates					
			Galactose	Rhamnose	Lactose	Sorbitol	Mannose	Maltose
L. fermentum	-	-	-	-	+	-	-	-
L. plantarum	-	-	-	-	+	+	+	+

Table 2. Antagonistic activity of isolates probiotic bacteria.

Probiotic bacteria	*E.coli* O157:H7						
	Well diffusion agar (mm)	(mm) Disk diffusion agar			Pour plate method in period of time CFU × 10^5		
		100	150	250	4h	12h	24h
L. fermentum	5	5	7	5	63.65	30.58	13.47
L. plantarum	8	7	7	8	137.5	57.57	20.62
B. bifidum	8	7	7	8	143	58.02	22

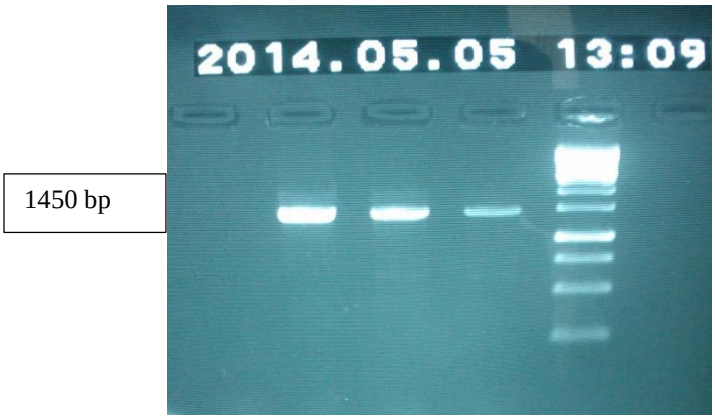

Figure 1. PCR amplification of 16srRNA gene in isolated bacteria. M: 1 Kb DNA ladder, P: Positive Control, 1: *Lactobacillus plantarum*, 2: *Lactobacillus fermantum*.

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

The authors gratefully acknowledge the support provided by Research council of Islamic Azad University of Rasht.

Conflict of interest

No conflict of interest associated with this work.

References

1. Rufter J. lactic acid bacteria and cancer. J *Nutrition* 2002; **88**: 589-594.
2. Çaglar E, Kargul B, Tanboga I. Bacteriotherapy and probiotics' role on oral health. *Oral Diseases* 2005; **11**(3): 131-137.
3. Kazemi Darsanaki R, Issazadeh K, et al. Antimutagenic activity of *Lactobacillus* spp.

isolated from fresh vegetables against sodium azide and 2-Nitrofluorene. *J Pure Appl Microbio* 2012; **6**: 1677-1682.
4. Dos Reis SA, da Conceição LL, Siqueira NP, et al. Review of the mechanisms of probiotic actions in the prevention of colorectal cancer. *Nutr Res* 2017; **37**: 1-19.
5. Day AS, Keenan JI. Probiotic-mediated modulation of host inflammation. *Expert Rev. Gastroenterol. Hepatol* 2011; **5**(3): 319-321.
6. Ng SC, Hart AL, Kamm MA, et al. Mechanism action of probiotics: recent advances. *Inflamm Bowel Dis* 2008; **15**(2): 300-305.
7. Dimidi E, Christodoulides S, Scott SM, et al. Mechanisms of action of probiotics and the gastrointestinal microbiota on gut motility and constipation. *Adv Nutr* 2017; **8**(3): 484-94.
8. De Roos NM, Katan MB. Effects of probiotic bacteria on human. *J Clin Nutr* 2000; **171**: 405-411.
9. Boris S, Jimenez-Diaz R, Caso JL, et al. Partial characterization of a bacteriocin produced by *Lactobacillus delbrueckii* subsp. *lactis* U0004, An intestinal isolate with probiotic potential. *J Appl Microbiol* 2001; **91**(2): 32-33.
10. Coconnier MH, Lievin V, Hemery E, et al. Antagonistic activity against *Helicobacter* infection in vitro and *in vivo* by human *Lactobacillus acidophilus* strain LB. *Appl Environ Microbiol* 1998; **64**: 4573-4580.
11. Ogunbanwo ST, Sanni AI, Onilude AA. Characterization of bacteriocin produced by *Lactobacillus plantarum* F1 and *Lactobacillus brevis* OG1. *Afr J Biotechnol* 2003; **2**(8): 219-227.
12. Ouwehand AC, Kirjavainen PV, Shortt C, et al. Probiotic: mechanisms and established effects. *Int Dairy J* 1999; **9**: 43-52.
13. Savadogo A, Cheik A. Antimicrobial activities of lactic acid bacteria strains isolated from Burkina Faso fermented milk. *Pak. J. Nutr* 2004; **3**(3): 174-179.
14. Kazemi Darsanaki R, Rokhi ML, Azizollahi-Aliabadi M, et al. Antimicrobial activities of *Lactobacillus* strains isolated from fresh vegetables. *Middle East J Sci Res* 2012; **11**(9): 1216-1219.
15. Strus M, Pakosz K, Gosciniak H. Antagonistic activity of *Lactobacillus* bacteria strains against anaerobic gastrointestinal tract pathogens *(Helicobacter pylori, Campilobacter coli, Campilobacter jejuni* and *Clostridium difficile). Med Dosw Mikrobiol* 2001; **53**(2): 133-142.
16. Sahshorpour M, Amani J, Jafari M, et al. Expression of recombinant chimeric EspA-intimin protein in *Nicotiana tobaccum* for oral vaccine development. *Journal of Genetic Plant Research* 2014; **1**(1): 77-94.
17. Ota A. Protection against an infectious disease by Enterohemorrhagic *Escherichia coli*. *Med Hypotheses* 1999; **53**(1): 87-88.
18. Zhao T, Doyle MP, Harmon BG, et al. Reduction of carriage of enterohemorrhagic *Escherichia coli* O157: H7 in cattle by inoculation with probiotic bacteria. *J Clin Microbiol* 1998; **36**(3): 641-647.
19. Gagnon M, Kheadr EE, Le Blay G, et al. In vitro inhibition of *Escherichia coli* O157: H7 by bifidobacterial strains of human origin. *Int J Food Microbiol* 2004; **92**(1): 69-78.
20. Brashears M, Jaroni D, Trimble J. Isolation, selection, and characterization of lactic acid bacteria for a competitive exclusion product to reduce shedding of *Escherichia coli* O157: H7 in cattle. *J Food Prot* 2003; **66**(3): 355-363.
21. Matsusaki H, Endo N, Sonomoto K, et al. Lantibiotic nisin Z fermentative production

by *Lactococcus lactis* IO-1: relationship between production of the lantibiotic and lactate and cell growth. *Appl Microbiol Biotechnol* 1996; **45**(1-2): 36-40.
22. Chakoosari MM, Faezi Ghasemi M, Masiha A. Antimicrobial effect of lactic acid bacteria against common pathogenic bacteria. *Bull. Env. Pharmacol. Life Sci* 2015; **9**(5): 1-4.
23. Sánchez B, Delgado S, Blanco-Míguez A, et al. Probiotics, gut microbiota, and their influence on host health and disease. *Mol Nutr Food Res* 2017; 61(1): 1-15.
24. Iglesias MB, Abadias M, Anguera M, et al. Antagonistic effect of probiotic bacteria against foodborne pathogens on fresh-cut pear. *LWT- Food sci. technol* 2017; **81**: 243-249.
25. Jørgensen MR, Kragelund C, Jensen PØ, et al. Probiotic *Lactobacillus reuteri* has antifungal effects on oral *Candida* species in vitro. *J Oral Microbiol* 2017; **9**(1): 1-8.
26. Kang MS, Lim HS, et al. Antimicrobial activity of *Lactobacillus salivarius* and *Lactobacillus fermentum* against *Staphylococcus aureus*. *Pathog Dis* 2017; **75**(2): 1-10.
27. Shu Q, Gill HS. A dietary probiotic (*Bifidobacterium lactis* HN019) reduces the severity of *Escherichia coli* O157: H7 infection in mice. *Med Microbiol Immun* 2001; **189**(3): 147-152.