Effect of consumption of fermented milk with Lactobacillus Casei and Lactobacillus Plantarum isolated from Ligvan Cheese against E.Coli O157:H7 Induced Infections in BALB/C Mice

Hamid Mirzaei[1*], Hamed Shahirfar[2], Haedeh Mobaiyen[3]

1- Faculty of Veterinary Medicine, Department of Food Hygiene, Tabriz Branch, Islamic Azad University, Tabriz, Iran
2- Faculty of Basic sciences, Department of Microbiology, Zanjan Branch, Islamic Azad University, Zanjan, Iran
3- Faculty of Medical Sciences, Department of Microbiology, Tabriz Branch, Islamic Azad University, Tabriz, Iran
*** Corresponding Author's Email:** hmirzaii@yahoo.com, hmirzaei@iaut.ac.ir

Abstract: In the recent years, association of *E.coli O157:H7* with hemorrhagic Colitis has been reported and this strain is known as causative agent of bloody diarrhea and predominant cause of hemolytic uremic syndrome (HUS). The aim of present study was to assess prevention role of fermented milk on *E.coli* O157:H7 infection which prepared by adding isolated *L.plantarum* (MLp)and *L.casei* (MlLc) from local Ligvan cheese either with only one probiotic or combined form of them (MLpc). In this study, 40 male mice (BALB/c strain) with an average of 6-8 weeks old were categorized in 4 groups. During a week, 1 (MLp), 2 (MLc), and 3 (MLpc) test groups were prescribed daily by 0.5 ml milk that were fermented by one of probiotic, the last group, was control group with normal nutrition. All of groups were fed by *Escherichia coli* O157:H7 equal 0.5 McFarland standards by using gavages, after 24 h from last treatments. The stool samples were taken from all of the group in days 1, 3, 5, and 7 in order to assess the excretion rate of *Escherichia coli* O157:H7. Five mice from each group were autopsied in 3rd and 7th day for evaluation of the colonization rate. So, 5cm of large intestine of mice were cut and serial dilutions of stool samples were prepared and cultured in Sorbitol Mac Conkey agar. White colonies (Sorbitol negative) in Sorbitol Mac Conkey agar were counted. Based on the statistical tests, there were significant difference ($P<0.05$) between test groups. The results of present study were shown that the consumption of fermented milk by probiotics could reduce significantly the exertion and colonization of *Escherichia coli* O157:H7 in the intestine of the mice.
[Hamid Mirzaei, Hamed Shahirfar, Haedeh Mobaiyen. **Effect of consumption of fermented milk with Lactobacillus Casei and Lactobacillus Plantarum isolated from Ligvan Cheese against E.Coli O157:H7 Induced Infections in BALB/C Mice.** *Life Sci J* 2012;9(4):5895-5898] (ISSN:1097-8135). http://www.lifesciencesite.com. 882

Keywords: Lighvan cheese, milk fermented by probiotic, *Lactobacillus plantarum, Lactobacillus casei*, *E.coli O157:H7*

1. Introduction

Escherichia genus consists of 6 species which *E.coli* has more importance. In the recent years the incidence of hemorrhagic colitis was associated with strains of *E.coli* O157:H7 so this strain is known as the cause of dysentery and hemolytic-uremic syndrome (HUS) (1, 2). The most important virulence factor for *E.coli* O157:H7 primarily is production of one or more Shiga-toxin which is called verotoxin (3, 4). Various medicines are used for treatment of *E.coli* O157:H7 infection, but this is a real fact that, application of drugs against Shiga-toxin producing bacteria, not only does not treat complication but increase toxin releasing and renal failures. So, hemolytic uremic syndrome occurs most commonly (2). In recent decades, according to several studies about probiotics that carried out in- vitro and in-vivo on human populations and laboratory animals showed very valuable properties such as treatment of intestinal resistance pathogens, prevention of viral and bacterial diarrhea, inhibitory effect on colon cancer, prevention of bladder cancer, improving the immune system, inhibit bacteria growth in small intestine, treatment of urogenital tract infections, treatment of Helicobacter pylori infections, improve lactose intolerance, reduce cholesterol (5-10). According the latest definition about probiotics, they are alive non-pathogen microorganisms in the foods that if they taken into the body in sufficient amounts can have positive impacts on the host (11, 12). The first food containing microorganisms probably was fermented milk (13) which Mechennkof found it. He observed that Bolgarian farmers had long life probably because of they drunk fermented milk (14). Consumption of probiotics can associate with normal flora survive and balance in the gut and prevent intestinal infection (15, 16). Based on researches, *Lactobacillus plantarum* and *L.casei* are the most important species of *Lactobacillus* which play an important role in cheese processing (17). Thus, acquired some information about normal lactic flora of the traditional cheese is necessary for preparing the safe and standard starter with best quality by maintaining the essential features of the product (18). The main objective of present

study was assessed the effect of fermented milk consumption against *E.coli* O157:H7 Infections Induced in BALB/C mice which prepared by *L. Casei* and *L. plantarum* singly and along each other isolated from Ligvan Cheese.

2. Materials and methods

Forty healthy male 6-8 weeks-old BALB/c mice (about 30±5 g body weight) were purchased from Razi Institute, Karaj, Iran. All of them were maintained at room temperature at a natural photoperiod for 1 week before experiment execution. A commercial balanced diet and tap water were provided. Management and husbandry conditions were identical in all groups with 12/12 h light/dark cycle at 21±2°C. The mice were randomly divided into 4 groups (10 mice each) as following:

Control group (C): In this group, animals were contaminated with *E.coli* O157: H7 and then feeding by sterile milk and water.

Treatment group 1 (MLc): Infected like control group then treated with fermented milk by *Lactobacillus casei.*

Treatment group 2 (MLp): Infected like control group then treated with fermented milk by *Lactobacillus plantarum.*

Treatment group 3 (MLcp): Infected like control group then treated with fermented milk by both of *L. plantarum* and *L. casei* .

L. plantarum and *L.casei* strains were provided from traditional Ligvan cheese which already has been approved by phenotypic and genotypic methods. To activate these bacteria, the probiotics were cultured separately in MRS broth and was incubated for 48 h at 37 °C. Then, fermented milk prepared in 3 separate Erlenmeyer with 250 ml sterile milk by adding 5 CC of *L.plantarum,* 5 CC of *L.casei* and 5 CC of *L.plantarum* and *L.casei* (2.5 CC of each) into the Erlenmeyers, respectively. Then, samples were incubated in a incubator with 100rpm shakeing at 37˚C until the pH reaches to 80 degree of Dornic. These milks used as primary starter. Fermented milk with *L.plantarum* and *L.casei* and combinative of them, were prepared by adding 10 ml of each primary starter which surcharge into the 100CC sterile milk then incubated at 37°C to reach it's acidity to 80 degree Dornic. Each of milks fermented by mentioned method was gavaged to treatment groups at the dose of 0.5ml for 7 days (30). Microbial suspension of E.coli O157: H7 corresponded to a 0.5 McFarland standard ($1.5*10^8$ CFU/ml) were prepared. In the 8th day, all groups feeding by E.coil O157: H7 by the same way.

E. coli O157:H7 were achieved from microbiology laboratory of veterinary medicine faculty of Islamic Azad University Tabriz branch. It was confirmed by sub cultured in nutrient agar and Mac Conkey Sorbitol Agar, IMViC tests, and O157 antiserum.

Counting of fecal-excreted E. colis O157:H7

In this term, on days 1, 3, 5 and 7 fecal samples were obtained from mice. Serial dilution of 10^{-1}- 10^{-6} of samples was prepared and surface plate count method were done from 3 last dilutions in the MacCONKEY sorbitol agar and were inocubated at 37˚C for 24 h (Zhao et al., 1998). The number of negative sorbitol colonies were counted and total number of E. coli O157:H7 were measured as following formula.

N= No. of suspicious colonies × reverse of the related dilution × Proportion of positive colonies revealed by antiserum

For assessment of colonization of E. coli O157:H7, it was carried on 5 mice in each group. For this, 5 cm of large intestine was took and after cleaning of its content, it sliced into the small spaces then washed in the 5cc normal saline and serial dilution were prepared and cultured like above method. They were incubated for 24-48 hours at 37°C. In addition to the above test, each group of mice was evaluated daily from clinical signs.

Statistical analysis

The statistical package for social sciences (SPSS Inc., Chicago, IL, USA), was used for statistical analysis. Data obtained were tested by ANOVA followed by Tukey's post-hoc multiple comparison test

3. Results

Results of the mean number of excreted *E.coli O157: H7* in different days of study are shown in Table 1. Based on results, it seen that the excretion of *E.coli O157: H7* in the treatment groups has decreased significantly in compared with control groups. Also, it shown that maximum excretion rate on days 1, 3, 5 and 7 is related to control group. Minimum rate on day 7 was associated with group treated by MLp and on days 1, 3, and 5 was related to group treated with MLc.

Results of the mean number of colonized *E.coli O157: H7* on 3rd and 7th days is shown in Table 2. Based on the obtained results it was observed that the colonization rate of E.coli O157: H7 in treated groups has decreased significantly than control groups. There was also observed that, the maximum colonization rate of *E.coli O157: H7* on days 3 and 7 is in the control group (C) and minimum rate on 3rd day was associated with group treated with MLc and on 7th day was related to group treated with MLp. It should be noted that there was no clinical signs observation in all groups.

Control group without fermented milk, MLc; Treamented group by fermented milk prepared by L. casei, MLp; Treamented group by fermented milk

prepared by L. plantrum, MLcp, Treamented group by fermented milk prepared by L. casei, and L. plantrum.

Table 1: Results of mean number of excretion rate of *E.coli O157: H7* on days 1, 3, 5 and 7 of study in terms of CFU / gr in the stool

Day / Group	1	3	5	7
C	$36\pm0.37\times10^{7\,a}$	$170\pm0.14\times10^{6\,a}$	$190\pm0.04\times10^{5\,a}$	$300\pm0.23\times10^{4\,a}$
MLc	$2.2\pm0.23\times10^{7\,b}$	$2.3\pm0.35\times10^{6\,b}$	$3.3\pm0.67\times10^{5\,b}$	$4.2\pm0.19\times10^{4\,b}$
MLp	$3.1\pm0.28\times10^{7\,b}$	$4\pm0.64\times10^{6\,b}$	$13\pm0.71\times10^{5\,b}$	$1.8\pm0.14\times10^{4\,b}$
MLcp	$2.2\pm0.24\times10^{7\,b}$	$2.8\pm0.34\times10^{6\,b}$	$6.2\pm0.32\times10^{5\,b}$	$2.6\pm0.36\times10^{4\,b}$

a,b,c: Dissimilar letters indicate significant differences in each column ($P<0.05$).C; Control group without fermented milk, MLc; Treamented group by fermented milk prepared by L. casei, MLp; Treamented group by fermented milk prepared by L. plantrum, MLcp, Treamented group by fermented milk prepared by L. casei, and L. plantrum

Table 2: Results of mean colonized rate of *E.coli O157: H7* in the large intestine based on CFU/cm^2

Group / Day	C	MLc	MLp	MLcp
3	$500\pm0.28\times10^{7\,a}$	$2.9\pm1\times10^{7\,b}$	$3.7\pm0.11\times10^{7\,b}$	$19\pm0.1\times10^{7\,b}$
7	$3.2\pm1.2\times10^{8\,a}$	$12\pm0.42\times10^{5\,b}$	$0.69\pm1.3\times10^{5\,b}$	$6.3\pm0.46\times10^{5\,b}$

a, b: Dissimilar letters indicate significant differences in each column ($P<0.05$).

4. Discussion and conclusion

The use of probiotics is back to when that people were starting to take fermented foods (19, 20). When the probiotics used as alive microorganisms, they can counteract with pathogenic microbial agent, can be immunized a person against the pathogens (21). Also, the previous studies in animals have shown that some commercial probiotic strains can be increased resistant against colonization and infection by pathogenic bacteria (22). In the present study, the effect of isolated *Lactobacillus plantarum* and *Lactobacillus casei* from Ligvan cheese were examined on excreting and colonization rate of *E.coli O157: H7* by using either with single probiotic or combined form of them, in BALB/c mice. Based on our results, fermented milk had inhibitory effects on colonization and excreting of *E.coli O157: H7* and could decrease severity and period of infection. The result of our study is compatible with other researches results. In Midolo et al., 1995 study which were done on the *Lactobacillus acidophilus*, *Lactobacillus casei* subspecies *rhamnosus*, showed that the probiotics can inhibit *Helicobacter pylori* growth isolated from clinical samples in in-vitro conditions (23). Kabir et al., 1997 studied on Inhibitory effects of *Lactobacillus salivarius* on *H. pylori* colonization in BALB/c mice and have concluded that *L. salivarius* has preventing effect on colonization of H. pylori in the stomach of the mice (24). Melanie et al during a research were studied inhibitory effects of some strains *Bifidobacteria* on *E.coli O157: H7*. Their results indicated that the inhibitory factor of *Bifidobacteria* was prevented of *E.coli* binding to Caco-2 cells (25). Gagnon et al., 2006 investigated about the effects of probiotic *B. thermoacidophiles* RBL-71 on *E.coli O157:H7* infection on BALB/c mouse. They found that consumption of probiotic can greatly reduce infections (26). Ota et al., 1999 reported that consumption of yogurt makes up more Lactobacillus colonization in the intestine which prevented colonization of Enterohemorrhagic E.coli (27). Lee et al., 2003 during an experiment found that *L.casei* of Vata milk prevents attachment of gastrointestinal bacteria to Caco-2 cells surface in 46% of cases. They also showed that maximum inhibitory effect was on *E.coli* TG1, *S.typhimurium* E10, *E.coli* ATCC 1775 and *S.typhimurium* ATCC 14028(>30%) (28). Aiba et al., 1998 showed that *lactobacillis* can reduce the colonization rate of *H.pylori* in the gastrointestinal tract (29). Carey et al., 2008 showed that probiotics have inhibitory effect on gene expression of Shiga-toxin 2 produced by *E.coli O157:H7* (30). Hirano et al., 2003 demonstrate that *Lactobacillus rhamnosus* has inhibitory effect in- vitro on enterohemorrhagic *E.coli* infection which were studied on human intestinal cells (31). The study of Lema et al., 2001 indicates that supplementing infected with E. coli O157:H7 lambs with Streptococus. *faecium* or a mixture of *S. faecium*, *L. acidophilus*, *L. casei*, *L. fermentum* and *L. plantarum* in the diet can reduce total number of *E. coli O157:H7* shedding in the feces and improve animal meat production performance as well (32). Based on present study can claim that consumption of fermented milk with probiotics has inhibitory effects on excretion and duration of disease caused by E.coli O157:H7.

Considering the results of present study, can be conclude that consumption of milk fermented with

L.plantarum and *L.casei* as single or combinative, results in decreasing of excretion and colonization rate of *E.coli O157:H7* in mice; that indicates we can apply some changes in the these two strains which made them as best quality starter in the production of local cheese.

Acknowledgement

Authors declare own thankful from colleges in faculty of medicine, Islamic Azad University, Tabriz branch.

References

1. Kovacs J, Roddy J, Gregoire S. Thrombotic thrombocytopenic purpura following hemorrhagic colitis due to Escherichia coli O157:H7. Am J Med 1990;88:177-179.
2. Molbak K, Mead PS, Griffin PM. Antimicrobial therapy in patients with Escherichia coli O157:H7 infection. J Am Med Assoc 2002;288:1014-1016.
3. Cimolai N, Bassalygas, Mah DG, Morrison BJ, Corter JE. A continuing assessment of risk factors for the development of E. coli O157:H7. associated hemalytic uremic syndrome. Clin Nephrol 1994;42:85-89.
4. Slutskerl Riec AA, Greene KD, Wells JG, Hutwagner L, Griffin PM. E.coli O157:H7 diarrhea in The USA. Clinical and epidemiological feature: Ann Intern Med 1997;126:505-13.
5. Aloja B, Radomira N, Dagma M, Peterv G. The possibilities of potentiating the efficacy of probiotics. Trend Food Sci Thechnol 2002;13:121- 126.
6. Aso Y, Akaza H. BLP study group. Prophylactic effects of lactobacillus casei preparation on the recurrence of superficial bladder cancer. Uro Int 1992;49:1125-1129.
7. Chapman MH, Sanderson IR. Intestinal flora and the mucosal immune system. Annales nestle 2003;1:55- 65.
8. Coconniier MH, lirvin V, Hemery E, Servin AL. Antagonistic activity against Helicobacter infection in vitro and in vivo by the human Lactobacillus acidophilus strain. L.B.APPL Environ Microbial 1998;64:4573-4580.
9. Michetti P, Dorta G, Wiesel PH, Brassart D, Vedu E. Effect of whey-based culture supernatant of lactobacillus acidophilus (johnsoni) La1 on Helicobacter pylori infection in humans. Digestion 1999;60:203-209.
10. Mirzaei H. Probiotics and introduction to their role in human health. 1st ed. Tabriz Islamic Azad University, 2004;1-2 (in Persian).
11. Araya M, Morelli L, Reid G, Sanders ME, Stanton C, Pineiro M. Guidelines for the evaluation of probiotics in food. Joint FAO/WHO Working Group Report on Drafting Guidelines for the Evaluation of Probiotics in Food, London. 2000;30:1–11.
12. Collado MC, Meriluoto J, Salminen S. In vitro analysis of probiotic strain combinations to inhibit pathogen adhesion to human intestinal mucus. Food Research International 2007;40(5):629-636.
13. Fernandez MF, Boris S, Barbes C. Probiotic properties of human lactobacilli strains to be used in the gastrointestinal tract. J Appl Microbiol 2003;94:449-455.
14. Brown AC, Valiere A. Probiotics and medical nutrition therapy. NIH Public Access 2004;7(2):56–68.
15. Bielecka M, Biedrzycka E, Biedrzycka E, Smoragiewicz W, Smieszek M. Interaction of Bifidobacterium and salmonella during associated growth. Int J Food Microbial 1998;45:151-155.
16. Kim S, Yang SJ, Koo HC, Bae WK, Kim JY, Park JH, et al. Inhibitory activity of Bifidobacterium longum HY8001 against vero cytotoxin of Escherichia coli O157:H7. J Food Protec 2001;64:1667-1673.
17. Garabal IJ, Alonsa PR, Acenteno J. Characterization of lactic acid bacteria isolated from raw cows' milk cheeses currently produced in Galicia (NW Spain). LWT- Food sci technol 2008;41:1452-1458.
18. Durul-Ozakaya F, Xanthopoulos V, Tunail N, Tzanetaki EL. Technologically important properties of lactic acid bacteria isolates from beyaz cheese made from raw ewe's milk. J Appl Micro 2001;91:861-870.
19. Laws A, Gu Y, Marshall V. Characterisation and design of bacterial exopolysaccharides from lactic acid bacteria. Biotechnol 2008;19(8):597-625.
20. Yamaguchi Y, Hearing VJ, Itami S, Yoshikawa K, Katayama I. Mesenchymal-epithelial interactions in the skin: aiming for site-specific tissue regeneration. J Dermatol Sci 2009;40(1):1-5.
21. James C. Diarrhea Caused by Escherichia Coli, Control of Communicable Diseases. Am Public Health Assoc 2000;155-158.
22. Silva AM, Barbosa FHF, Duarte R, Vieira LQ, Arantes RME, Nicoli JR. Effect of Bifidobacterium longum ingestion on experimental Salmonellosis in mice. J Appl Microbiol 2004;97:29-37.
23. Midolo PD, Lambert JR, Hull R, Luo F, Grayson ML. In vitro inhibition of Helicobacter pylori NCTC 11637 by organic. J Appl Microbiol 1995;79(4):475-479.
24. Kabir AM, Aiba Y, Takagi A. Prevention of Helicobacter pylori infection by lactobacilli in a gnotobiotic murine model. Gut 1997;41(1):49-55.
25. Melanie G, Ehab EK, Gwenaelle LB, Ismail F. In vitro inhibition of Escherichia coli O157:H7 by bifidbacterial strains of human origin. Int J food Microbial 2004;92:69-78.
26. Gagnon M, Kheadr EE, Dabour N, Richard D, Fliss I. Effect of Bifidobacterium thermacidophilum probiotic feeding on enterohemorrhagic Escherichia coli O157:H7 infection in BALB/c mice. Int J Food Microbiol 2006;111(1):26-33.
27. Ota A. Protection against an infectious disease by Enterohemorrhagic Escherchia coli. Med Hypotheses 1999;53(1):87-98.
28. Lee YK, Puong KY, Ouwehand A, Salminen S. Displacement of bacterial pathogens from mucus on Caco-2 cell surface by lactobacilli. J Med Microbial 2003;52:925-930.
29. Aiba Y, Suzuki N, Kabir AM, Takagi A, Koga Y. Lactic acid-mediated suppression of Helicobacter pylori by the oral administration of lactobacillus salivarius as a probiotic in a gnotobiotic murine model. AM J Gasteroenterol 1998;93(11):2097-2101.
30. Mirzaei H, Nahaei MR, Taheri SA, Akbari Nakhjevani S. The effect of Bifidobacterium Angulatum Fermented Milk on Escherichia coli O157:H7 infection in mice. Medical J Tabriz Uni Med Sci Health Services 2010;32(1):80-85.
31. Hirano J, Yoshida T, Sugiyama T, Koide N, Mori I, Yokochi T. The effect of Lactobacillus rhamnosus on enterohemorrhagic Escherichia coli infection of human intestinal cells in vitro. Microbiol Immunol 2003;47(6):405-9.
32. Lema M, Williams L, Rao DR. Reduction of fecal shedding of enterohemorrhagic Escherichia coli O157:H7 in lambs by feeding microbial feed supplement. Small Rumin Res 2001;39(1):31-39.