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Original Article

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

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ABSTRACT
Escherichia coli is one of the most important species in the Escherichia genus. In the recent years hemorrhagic Colitis has been associated with au strain called E. coli O157:H7 and this strain is known as causative agent of bloody diarrhea and predominant cause of hemolytic uremic syndrome (HUS). The main objective of present study was to prevent of E.Coli O157:H7 infection both directly and indirectly by using of L.plantarum and L.casei isolated from Ligvan cheese as single and combined use. In this study 40 mice of 6-8 weeks old were divided into 4 groups of 10 mice by chance. Stool of mice studied for recovery of E. coli O157:H7 before getting infected and on days 3,5 and 7 after getting infected with the test organism. For identification of E. coli O157:H7 MacConkey sorbitol agar was used and for confirmation of the diagnosis specific antiserum against E. coli O157 was employed. Results showed that the average excretion of E.Coli O157: H7 in the treatment groups has decreased significantly compared with control groups. It was observed that the average excretion of E.Coli O157: H7 in the first day in the group treated with Lactobacillus casei (MLc) has significant difference with all groups except the group treated with Lactobacillus casei and Lactobacillus plantarum (MLcp). Also we found that the average colonization E.Coli O157: H7 in treated groups has decreased significantly than control groups. There was also observed that, the highest average colonization E.Coli O157: H7 on days 3 and 7 is in the control group (C), but unlike the results it seen on day 3 and 7 that the lowest average E.Coli O157: H7 on day 3 is in the group treated with lactobacillus casei (MLc) and on day 7 is in the group treated with of Lactobacillus plantarum and Lactobacillus casei (MLcp). Consumption of milk fermented by L. casei and L. plantarum minimizes the duration of illness and reduces the severity of the illness. Further studies are needed on humans.
Keywords: *L.casei, L.plantarum, E.Coli O157:H7, Ligvan cheese, infection, BALB/C Mice.*

INTRODUCTION

Escherichia genus consists of 6 species so that *E.Coli* has more importance. In 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, but, the fact is that, use of drugs against Shiga-toxin producing bacteria, not only does not treat complication but yields to increase toxin releasing and renal failures. So, hemolytic uremic syndrome occurs most commonly [2]. In recent decades, according to several studies that carried out in In vitro and In vivo condition of human populations and laboratory animals, very valuable properties such as resistance against intestinal pathogens treatment and prevention of viral and bacterial diarrhea, inhibitory effect on colon cancer, prevention of bladder cancer, improving the immune system, inhibit bacteria growth of small intestine, treatment of urogenital tract infections, treatment of infections caused by Helicobacter pylori, improve lactose intolerance, reduce cholesterol, etc. are attributed to probiotics [5-10]. Based on latest definition about probiotics, they are alive non-pathogen microorganisms in the foods that If taken into the body in sufficient amounts can have a positive impacts on the host [11,12]. It was probably that the first food containing live microorganisms was fermented milk [13,14]. Consumption of probiotics can associate with flora survive and balance in the gut and prevent intestinal infection [15,16]. Based on researches done,

L.plantarum and L.casei are the most important species of Lactobacillus which play an important role in cheese processing [17]. Thus, understanding of the normal flora composition of the traditional cheese provides the preparing the starter for producing a safe and standard compound with maintaining the essential features of the product [18]. The main objective of present study was beneficiates 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.

MATERIALS AND METHODS

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

Control group (C): In this group, animals were contaminated with *E.Coli* O157: H7 and water and food intake was without probiotic Lactobacillus plantarum and Lactobacillus casei in it.

Treatment group 1 (MLc): infected like control group then treated with lactobacillus casei.

Treatment group 2 (MLp): infected like control group then treated with lactobacillus plantarum.

Treatment group 3 (MLcp): infected like control group then treated with both of lactobacillus plantarum and lactobacillus casei [24].

Lactobacillus plantarum and Lactobacillus casei were provided from folk Ligvan cheese which already has been approved by phenotypic and genotypic methods.

To activate these, the probiotics were cultured separately in MRS broth and was incubated for 48 h at 37 °C. Then, for preparation of fermented milk, in 3 separate Erlenmeyer with 250 ml sterile in each; we added 5 cc *L.plantarum*, 5cc *L.casei* and 5cc *L.plantarum* and *L.casei* (2.5cc of each) into the Erlenmeyers, respectively. Then, Erlenmeyers were incubated in a shaker incubator at 37˚C until the pH reaches to 80 degree Dornic. These milks used as primary starter. But, for preparing the fermented milk with *L.plantarum* and *L.casei* and combinative of them, 10 ml of each primary starter was added into the 100cc sterile milk then incubated at 37°C to reach it 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].

E. coli O157:H7 were achieved from microbiology laboratory of veterinary medicine faculty of Tabriz University. For verification of strain O157:H7 by culturing in nutrient agar culture media and testing of IMViC, O157 antiserum was confirmed. Then from colonies, concentration of 0.5 which had 1.5×108 CFUg-1 was prepared based on Mac Farland scale. For feeding the bacteria *E.Coli* O157: H7 to mice, 24 hours after inoculation of fermented milk with probiotic, this was given as gavage to all 4 groups at the 5/0 ml [31, 24].

Counting of fecal-excreted E. colis O157:H7

In this term, on days 1, 3, 5 and 7 fecal samples was obtained from mice. Concentrations of 10-1, 10-2, 10-3, 10-4, 10-5 and 10-6 prepared and from 3 last dilutions surface culture were exerted in the macconkey sorbitol agar and were inoculated at 37˚C for 24 h (Zhao et al., 1998). Then amount of negative sorbitol colonies were counted and amount 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 out 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. Then, after preparation of dilution from solution, 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.

RESULTS

Results of the average count of *E.Coli* O157: H7 excreted and days of excretion 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 compared with control groups. Also, it shown that maximum and minimum excretion rate on days 1,3,5 and 7 is related to control and treatment groups respectively. 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 counting of *E.Coli* O157: H7 colonization and days of excretion 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 day 3 was associated with group treated with MLc and on day 7 was related to group treated with MLp. It should be noted that there was no observed clinical signs in groups.

Table 1: results of excrete rate of *E.Coli* O157: H7 on days 1, 3, 5 and 7 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 ($P<0.05$).

Table 2: results obtained from colonization rate of *E.Coli* O157: H7 in the large intestine based on CFU/cm^2

Group / Day	C	LC	LP	LCP
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 ($P<0.05$).

DISCUSSION AND CONCLUSION

The use of probiotics is the time that people were starting to fermented foods [20,19]. Using probiotics as live microorganisms that can counteract with pathogenic microbial agent, can be immunized a person against the pathogens [21]. Also, previous studies in animals have shown that some commercial probiotic strains can be increased resistant against colonization and infection by bacteria pathogenic [22]. In the present study which was conducted in in-vivo conditions, the effect of Lactobacillus plantarum and Lactobacillus casei isolated from Ligvan cheese were examined both single and combined use on excreting and colonization rate of *E.Coli* O157: H7 in BALB/c mice. The result of our study is compatible with other researches results, so that, Midolo et al., 1995 with a study on the species Lactobacillus acidophilus, Lactobacillus casei rhamnosus, showed that the probiotics can inhibit growth of clinical isolates of Helicobacter pylori in vitro conditions [23]. Kabir et al. studied on Inhibitory effects of Lactobacillus salivarius on *H. pylori* colonization in BALB/c mice and have concluded that Lactobacillus 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 indicate that the inhibitory factor of bifidobacteria on *E.Coli* is prevention of *E.Coli* binding to Caco-2 cells [25]. Gagnon et al., investigated effects of probiotic Bifidobacterium thermoacidophiles RBL-71 on BALB/c mouse infected with Escherichia coli O157: H7 and found that consumption of probiotic Bifidobacterium thermoacidophiles RBL-71 can greatly reduce infections of *E.Coli* O157: H7 in the intestines of mice, BALB/c [26]. Ota *et al*. reported that consumption of yogurt makes up more Lactobacillus colonization in the intestine and Conditions that prevent colonization of enterohemorrhagic *E.Coli* [27]. Lee et al., during an experiment found that L.casei shirota in 46% of cases prevents attachment of gastrointestinal bacteria to Caco-2 cells surface. They also showed that maximum

inhibitory effect of L.casei shirota (>30%) was on *E.Coli* TG1, S.typhimurium E10, *E.Coli* ATCC 1775 and S.typhimurium ATCC 14028 [28]. Aiba et al. showed that lactobacillis can reduce the colonization rate of H.pylori in the GI 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., demonstrate that Lactobacillus rhamnosus has inhibitory effect on enterohemorrhagic *E.Coli* infection of human intestinal cells in vitro [31]. The study of Lema et al., 2001 indicates that supplementing lambs infected with E. coli O157:H7 with S. 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 shed 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 rats; that indicates can apply some changes in the these two strains to use of them as starter in the production of local cheese.

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