Prevention of Infections Produced by *Escherichia coli* and *Listeria monocytogenes* by Feeding Milk Fermented With Lactobacilli

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**ABSTRACT**

Challenge studies were set up feeding *Lactobacillus casei* and *Lactobacillus acidophilus* fermented milk and two different pathogenic microorganisms: *Listeria monocytogenes* and enteroinvasive *Escherichia coli*. Mice were fed for 8 consecutive days with fermented milk and then challenged with the pathogens. The survival rate in control mice was 62% for *Listeria* and 83% for *E. coli*, while 100% protection was observed for the 20 d per vial in treated mice. Colonization of the liver and spleen by *E. coli*, while 100% protection was observed for the 20 d per vial in treated mice. Colonization of the liver and spleen by *E. coli*, while 100% protection was observed for the 20 d per vial in treated mice. Colonization of the liver and spleen by *E. coli*, while 100% protection was observed for the 20 d per vial in treated mice. Colonization of the liver and spleen by *E. coli*, while 100% protection was observed for the 20 d per vial in treated mice. Colonization of the liver and spleen by *E. coli*, while 100% protection was observed for the 20 d per vial in treated mice. Colonization of the liver and spleen by *E. coli*, while 100% protection was observed for the 20 d per vial in treated mice. Colonization of the liver and spleen by *E. coli*, while 100% protection was observed for the 20 d per vial in treated mice. Colonization of the liver and spleen by *E. coli*, while 100% protection was observed for the 20 d per vial in treated mice. Colonization of the liver and spleen by *E. coli*, while 100% protection was observed for the 20 d per vial in treated mice. Colonization of the liver and spleen by *E. coli*, while 100% protection was observed for the 20 d per vial in treated mice. Colonization of the liver and spleen by *E. coli*, while 100% protection was observed for the 20 d per vial in treated mice. Colonization of the liver and spleen by *E. coli*, while 100% protection was observed for the 20 d per vial in treated mice. Colonization of the liver and spleen by *E. coli*, while 100% protection was observed for the 20 d per vial in treated mice. Colonization of the liver and spleen by *E. coli*, while 100% protection was observed for the 20 d per vial in treated mice. Colonization of the liver and spleen by *E. coli*, while 100% protection was observed for the 20 d per vial in treated mice.

The lactic acid bacteria inhabit various ecosystems, widely distributed in nature, including human and animals (13), and in agricultural products (30). They are also used in the production of yogurt, lactic acid beverages, cheese, fermented butter, soy sauce, sake, pickles, and salami (30). They have also found use in pharmaceuticals such as lactic acid bacteria preparations and as additives in animal feed (5,9). Thus, specific kinds of lactic acid bacteria are involved in certain types of food processing, providing characteristic tastes or aromas (1).

The lactic acid bacteria which colonize in the intestine of humans and animals are indigenous to the intestine and differ from the lactic acid bacteria found in food (11). From birth, these bacteria start to proliferate on the skin and on the mucosa of the different tracts (32). At the first day of life, an equilibrium is established in the intestine between the host and the intestinal bacteria which results in the formation of a normal intestinal bacterial flora (10,20). In many animals, lactobacilli are the predominant bacteria in intestine, while bifidobacteria and streptococci colonize others (7). The equilibrium reached is by no means permanent and may be disturbed by various factors. The factors which influence the intestinal flora can be different. These include the physical conditions of host, host immunity, diet, interaction between intestinal bacteria, or antibiotic treatments (2). Another important factor is the presence of pathogenic microorganisms, or their toxins, which can lead to an unbalanced bacterial flora, causing acute or chronic diseases (6,16,19).

Based upon these considerations, we isolated two strains of lactobacilli in our laboratory, namely, *Lactobacillus casei* and *Lactobacillus acidophilus*, from a human source. Both of these organisms are capable of surviving and becoming established in the human gastrointestinal tract. We have used milk fermented with these strains for the prevention and treatment of diarrhea in children (12). Also, the immunostimulant capability of these strains in mice has also been demonstrated (25). Feeding milk fermented with *L. casei* and *L. acidophilus* to mice resulted in an increased resistance to *Salmonella typhimurium* (26) and *Shigella sonnei* (21). We also have shown the inhibitory effect of these strains on the growth of *Listeria monocytogenes* (28), *Shigella sonnei* (3), *Escherichia coli*, and other enteropathogens (data not published).

*L. monocytogenes* can cause a series of different infectious diseases, as for example, meningocencephalitis, perinatal infections, endocarditis, and pneumonias. It has been recently associated with foodborne disease outbreaks (17).

Nomoto et al. (23) have shown the effect of heat killed *L. casei* intravenously administered on the augmentation of host resistance to *L. monocytogenes*. Sato et al. (29) demonstrated the role of macrophages in the enhancement of host resistance against this pathogen by *L. casei* administered by the same route. They have shown that the cells involved in the listeria endovenously challenged infection are the macrophages.

Another pathogen frequently isolated as a cause of diarrhea is *E. coli*, especially the enteroinvasive strains. *E. coli* is one of the predominant species among the facultative anaerobic normal flora of the intestine, but within this species, however, there are fully pathogenic strains that cause distinct syndromes of diarrheal disease (16). These have been classified by Levine (14) in four main categories.

As the feeding with fermented milk was effective in the *Salmonella* and *Shigella* challenged mice, the present study was designed to examine whether feeding mice with milk...
fermented with lactobacilli could also protect against infection with enteroinvasive *E. coli* and *L. monocytogenes*. This then could support the use of fermented milks for the treatment and prevention of many intestinal infections.

**MATERIALS AND METHODS**

**Animals**

Swiss albino mice weighing 25-30 g were obtained from the random bred colony kept by our department at CERELA. The animals were housed in plastic cages at room temperature. Each experimental group consisted of 20-30 mice (4-5 for each different day), housed individually during experiments.

**Microorganisms**

The two lactobacilli strains used were *L. casei* and *L. acidophilus* both isolated from human feces. Their isolation, maintenance, and preparation of fermented milk were described previously (12).

*L. monocytogenes* and *E. coli* were isolated from human pathogenic sources (blood and feces, respectively) in the Clinical Microbiological Department of the Biochemistry Faculty, National University of Tucumán. They were grown in brain heart infusion (Difco, Detroit, MI) at 37°C for 6 h and washed three times by centrifugation with saline solution before use.

**Feeding procedure**

Mice were fed daily for 8 consecutive days with milk fermented with *L. casei* and *L. acidophilus* containing 1.5 x 10⁹ CFU. The control group received 10% skim milk powder.

**Challenge**

After the 8-d feeding treatment, the mice were challenged orally with the pathogens introduced by an oral catheter. The group challenged with *L. monocytogenes* received 3 x 10⁹ CFU and the groups challenged with *E. coli*, 2.5 x 10⁹ CFU.

**Circulating antibodies**

Mice were bled from the retroorbital venous plexus. The sera were diluted and antibodies titers determined against lactobacilli, *L. casei*, and *E. coli* suspensions (3 x 10⁶ cells) by the tube agglutination test (22).

**Viable counts of pathogens in tissue homogenates**

The number of viable bacteria in the liver and spleen were determined both in the control and experimental groups on the days 1, 2, 5, and 7 postchallenge.

The methodology used was described previously (21). The medium used for the enumeration of *L. monocytogenes* was blood agar and for *E. coli*, McConkey agar (Difco).

**Antibodies from intestinal fluid**

The procedure for collection of intestinal fluid was a modification of that described by Lim et al. (15) for the isolation of intestinal mucosal lymphoid cells. Small intestines from each mouse were carefully removed from the gastric/duodenal junction and the ileal/caecal junction. The contents were washed out with 1 ml cold phosphate buffered saline (PBS, pH 7.2), centrifuged at 2000 g for 30 min. The supernatant fluid was retained for antibody determination. Antibody titers were determined by diluting the intestinal fluid in PBS and testing for agglutination of lactobacilli or pathogens as before. Antibodies were measured on the same day as the colonization assays.

**Determination of protection**

Treated and control groups which had been fed for 8 d were challenged with the different pathogens and observed for 20 d. The daily death count was recorded as percentage survival in each group.

**Statistical analysis**

Results were expressed as the arithmetic mean of number of values obtained ± Standard Deviation. The significance of the results was analyzed by Student’s t test.

**RESULTS**

Enhancement of resistance to pathogens by feeding fermented milk

After feeding for 8 consecutive days with fermented milk, mice were challenged with *L. monocytogenes* or with *E. coli*, and observed for survival for 20 d (Fig. 1). Treated mice showed a survival percentage of 100% in both groups, indicating complete protection with the fermented milk treatment, while the control groups showed a mortality of 17% for *E. coli* and 38% for *L. monocytogenes* by the 20th day.

![Figure 1. Survival rate of mice fed with fermented milk for 8 consecutive days and challenged orally with pathogens: ( ) Control mice fed with skim milk powder challenged with *L. monocytogenes* and *E. coli*. ( ) Treated mice fed with fermented milk and challenged with *L. monocytogenes*. ( ) Treated mice fed with fermented milk and challenged with *E. coli*. Results of two experiments, 10 mice each one. The bars represent the standard deviation of the mean.](http://meridian.allenpress.com/jfp/article-pdf/56/5/401/1664869/0362-028x-56_5_401.pdf)

Effect of fermented milk treatment on the growth of pathogens in liver and spleen

Viable *E. coli* in the liver of control mice began to appear by the 2nd day postchallenge, reaching the highest level on day 5 (Fig. 2b), maintaining the high level up to day 7 (10⁷ CFU per organ). The kinetics of appearance of *E. coli* in the liver of treated mice was different, because they appeared on the 2nd day, disappearing completely on the 5th day. The same type of kinetics was obtained in the spleen of control and treated mice but with values slightly lower than in liver. The pathogen also disappeared completely on the 5th day postchallenge in the spleen of treated mice (Fig. 2a).

In animals challenged with *L. monocytogenes*, the pathogen began to appear on the 1st day postchallenge in the livers.
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Days post-challenge

Figure 2. a) Number of E. coli in spleen of mice fed with fermented milk with L. casei and L. acidophilus for 8 consecutive days, then challenged with 2.5 x 10⁹ CFU of E. coli. (A - A) Control mice. (Δ - Δ) Treated mice. b) Number of E. coli in liver of the same mice. (O - O) Control mice. (● - ●) Treated mice. The bars represent the standard deviation of the mean.

of control and treated mice, following the same type of distribution but with lower values in the treated mice (Fig. 3b), and with a higher difference on the 7th day post-challenge. The same occurred in the spleen of both groups (Fig. 3a) but with a tendency to decrease the number of pathogens in both groups on the 7th day.

Circulating and intestinal antibodies

The levels of anti-E. coli antibodies from sera are shown in Fig. 4. Fivefold higher titers were obtained in the treated mice on day 5. The titers of antilactobacilli antibodies of both groups were between 10 and 20 (inverse dilution). The levels of anti-Listeria antibodies were 3 to 4 times higher than control, but lower than those obtained for the E. coli challenged mice. The levels of anti-E. coli antibodies from sera were comparable to the levels obtained from S. sonnei (21). Both E. coli and S. sonnei are like cell walls; it would expect the same response.

Titers of anti-E. coli antibodies from small intestinal fluid are shown in Fig. 5, obtaining differences statistically significant between the treated and control groups. The same results were obtained in the L. monocytogenes challenged groups, referred to the higher levels in the group treated with fermented milk.

DISCUSSION

The dominant autochthonous microorganisms can regulate the indigenous flora and can interfere with the pathogenic bacteria which cross the gut, by a number of mechanisms proposed by different groups of researches (5,18) and discussed by Fuller (9). This concept has lead to the inoculation of selected microbial strains to promote the resistance to infectious diseases, as cited by Tannock (5,31). The rationale
for the use of probiotics in man has been described also by Marteau et al. (18).

The products fermented specifically with lactobacilli have been extensively used for several purposes (30) as well as controlling diarrhea (8,12). In this study, we use two strains of lactobacilli isolated from human source, being capable of surviving and becoming established in the human gastrointestinal tract. We used milk fermented by them for the prevention and treatment of infantile diarrhea in children (12). We have demonstrated its protective effect against *Salmonella* (26) and *Shigella* infections (21). In this work, we describe the effect of feeding fermented milk on the prevention to *E. coli* and *L. monocytogenes* infections.

When animals were fed with milk fermented with *L. casei* and *L. acidophilus*, evidence of the protective effect to these infections was demonstrated. There were: a) Differences statistically significant (p < 0.005) were obtained between the control and treated groups in the resistance and survival rate: a protection of 100% against pathogen infection was produced; b) The colonization of liver and spleen in the *E. coli* challenged animals was better resolved, because on the 5th day there were no pathogens, indicating that the immunostimulant effect produced by fermented milk (25) involves those cells and lymphokines responsible for the complete elimination of the pathogen (4). In the *Listeria* challenged mice, the colonization followed similar kinetics in the treated and control mice, but with very significant differences (p < 0.005) in the number of colonies grown in liver and spleen. The difference between the *E. coli* and this group is that the pathogen never decreases to 0 levels on the days studied. These results could be explained by the different type of cells involved in the response to *Listeria* infection, the macrophages as described by Ohara (24) and Sato (29); c) The levels of antibodies in both sera and intestinal lavage were always enhanced by the treatment with fermented milk. The anti-*E. coli* antibodies were higher than the anti-*Listeria* antibodies, being in both cases 3- to 5-fold higher than their respective controls, supporting again the differences between the mechanisms involved in both infections, being the T and B lymphocytes mainly in the *E. coli* and the macrophages in the *Listeria* challenged mice.

The high level of sera antibodies is explained by the stimulation of the immune system by an oral antigen, supported by the work of Phillips-Quagliata et al. (27) who demonstrated the existence of a common mucosal immune system directly related with the systemic immune system. The higher level of antipathogens antibodies detected in the intestinal fluid is explained also by the concept of the review of Azim (4).

The higher resistance to *E. coli* and *Listeria* infections produced by feeding fermented milk shows that lactic acid bacteria have potential uses in the prevention of different infections.

**REFERENCES**


