Journal of Food Protection, Vol. 62, No. 12, 1999, Pages 1430–1434

Study of Adhesion of *Lactobacillus casei* CRL 431 to Ileal Intestinal Cells of Mice

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MS 99-75: Received 17 March 1999/Accepted 17 July 1999

ABSTRACT

It is well known that the cell wall of *Lactobacillus casei* CRL 431, a strain present in probiotics, presents lectinlike surface molecules. Presence of these molecules stimulates the immune system. Given the role that lectins and lectinlike substances play in the adhesion phenomenon, it is probable that this is an initial stage in the immunostimulation produced by this bacterium. To confirm this, adhesion of this microorganism to exfoliated mouse ileal epithelial cells was studied in vitro. Other *L. casei* strains isolated from adult human intestines and one of dairy origin were also examined for their ability to adhere to ileal epithelial cells. Another strain, which was included in the present study, was *Lactobacillus acidophilus* CRL 730. *L. casei* strains isolated from humans showed good ability to adhere to ileal epithelial cells, whereas *L. casei* isolated from dairy origin did not. Adhesion was only observed at 37°C and at a pH between 6 and 7.5. The exposure time needed for highest adhesion was 30 min. Presence of lectinlike substances on the surface of *L. casei* CRL 431 is important to this adhesion phenomenon, since adherence capacity was lost after removal of these substances.

Studies of colonization of human intestines by pathogenic bacteria have led to identification of a number of factors involved in adhesion and the genes related to this phenomenon *(7)*. On the contrary, little is known about microorganisms that constitute the indigenous flora, which is due to the ecosystem complexity and the difficulty to obtain mutants.

Lactic acid bacteria are common inhabitants of the human and animal gastrointestinal duct and therefore are considered beneficial. Although it has not been clarified yet whether adhesion is essential for a probiotic to become beneficial, it is a property that enhances bacterial survival.

During the last 2 decades, microbiologists have mainly focused on adhesion qualities of bacteria and other cells, and these characteristics have gained more and more importance in microbial ecology. Conway and Kjelleberg *(5)* and Henriksson et al. *(12)* demonstrated that proteins located on the bacterial surface are the determining factor in adhesion of a *Lactobacillus fermentum* strain to host-specific stomach epithelium from pigs and that carbohydrates were only partially involved. This may also occur in other microorganisms and could explain species specificity. Mukai et al. *(18)* determined a lectinlike substance in a *Lactobacillus acidophilus* strain through hemagglutination assays with human and animal erythrocytes. This agglutination activity diminished when bacterial cells were treated with proteinase K or glycoproteins, whereas presence of different types of monosaccharides and disaccharides did not affect activity.

Coconnier et al. *(3)* demonstrated that *L. acidophilus* BG2FO4 adheres to Caco-2 cells primarily through involvement of a secreted protein found in spent culture supernatant and secondarily through involvement of cell surface proteins and carbohydrate molecules. Chauvière et al. *(2)* found a cell surface factor that was not sensitive to protease and another heat-resistant, protease-sensitive extracellular factor. Furthermore, they found a substance in the supernatant of a *L. acidophilus* LB culture able to inhibit adhesion of *Escherichia coli* to Caco-2 cells. Greene and Klaenhammer *(11)* did not confirm the presence of a primary mediator of adherence of *L. acidophilus* BG2FO4 in spent culture supernatant; instead, adhesion to human intestinal cells involved different combinations of carbohydrate and protein factors on the bacterial cell surface. Recently, Adlerberth et al. *(1)* studied the efficiency of colonization of the intestines of the host under experimental conditions of two strains of *Lactobacillus plantarum* isolated from human beings. Adherence ability to human colon cell lines was correlated to agglutination with *Saccharomyces cerevisiae* and with erythrocytes. This bond was manose sensitive. These are only some of the numerous findings regarding adhesion of lactic acid bacteria to intestinal epithelial cells.

Previous studies performed in our laboratory have demonstrated that *L. casei* CRL 431 possesses external lectinlike structures that emerge from the cell surface. These molecules have the property to bind in a specific way to hydrocarbons, which would be present in intestinal mucin or on the surface of epithelial cells. Only when these molecules are present is the microorganism able to stimulate the immune system of mice *(15–17)*.

L. casei CRL 431 and *L. acidophilus* CRL 730, both strains of human origin and selected for probiotic proper-

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TABLE 1. *Characteristics of adhesion of lactobacilli to mouse IEC*[a]

Microorganism	% adhesion	Number of adhered bacteria/100 IEC
L. casei CRL 431 (human origin)	53.3 ± 6	55 ± 7
L. acidophilus CRL 730 (human origin)	12 ± 1.3	16 ± 1.5
L. casei CRL 575 (human origin)	52.4 ± 8	100 ± 11
L. casei CRL 55 (dairy origin)	8.33 ± 1.2	8.3 ± 0.9

[a] Data are given as mean ± SD.

ties, constitute a starter culture of a food product sold in the Mercosur market. In the present work, in vitro studies under different conditions were performed to determine highest adhesion of *L. casei* CRL 431 to ileal epithelial cells (IECs). Two more lactobacillus strains from human and dairy origin were also assayed.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *L. casei* CRL 431, *L. acidophilus* CRL 730, and *L. casei* CRL 575 are of human origin (feces of healthy children). *L. casei* CRL 55 is of local handmade cheese origin. All strains were obtained from the CERELA Culture Collection (Culture Collection of Centro de Referencia para Lactobacilos, Tucumán, Argentina) and grown at 37°C in LAPTg medium *(20)*. They were subcultured at least three times before the adhesion assay.

Animals. Six-week-old Balb/c mice, each weighing 25 to 30 g, were obtained from the random-bred colony kept at our center. The mice were kept in metal cages with natural illumination and had free access to feed and water. The animals were fed with a balanced diet. They fasted for 24 h before we performed isolation of IECs. The mice were sacrificed by cervical dislocation.

IEC preparation. A modified method according to Jin et al. *(13)* was used for preparing IECs. Epithelial cells of the terminal 5 to 10 cm of the ileum were scraped off gently with the edge of a microscope slide, and the scrapings were suspended in phosphate-buffered saline (PBS) containing NaCl (8 g/liter) and KCl (0.2 g/liter) (pH 7.4). The suspended scrapings were kept on ice for 15 min to allow large debris to settle. The debris was then removed, and the supernatant fluid centrifuged at 120 × *g* for 10 min to spin down the cells in suspension. The pellet, which contained the IECs, was washed twice with PBS, resuspended in NCTC 135 medium (Sigma Chemical Co., St. Louis, Mo.), supplemented with 10% inactivated fetal bovine serum, and diluted to a concentration of approximately 5×10^5 cells/ml. The number of viable cells in the preparation was determined by trypan blue staining. The fresh suspension contained approximately 85% of viable cells. The cell suspensions, stored at 4°C, were used within 3 h for adhesion studies.

In vitro adhesion assay. A modified technique according to Jin et al. *(13)* was used for determination of adhesion. A stationary-phase bacteria culture was centrifuged at 3,000 × *g*, and the pellet was washed twice with PBS and then diluted to a concentration of 2×10^7 cells using the same buffer solution. Suspensions of lactobacilli (0.2 ml) and IECs (0.8 ml) were poured together, and the mixture was incubated at 37°C for 1 h for all assays unless stated otherwise. After incubation, the mixtures were kept on ice. The number of bacteria adhered to IECs was counted from 50 μl of each mixture using phase-contrast microscopy. Thirty epithelial cells were examined at random, and the number of IECs with attached bacteria (adhesion percentage) and the mean number of bacteria attached per IEC, extrapolated to the number of cells adhered to 100 IEC, were calculated. Chains or pairs of bacterial cells were counted as one unit. The temperatures assayed were 4, 25, 37, and 42°C. Two suspension buffers for the bacteria were studied at pH values 4, 5, 6, 7, 7.5, and 8. Acetic acid and acetate buffer for pH values 4 and 5 and PBS for pH values 6, 7, and 8 were used. Effect of exposure times (30, 60, 120, and 180 min) on bacterial adhesion was also studied.

Biochemical characterization of adherence. The adhesion phenomena were characterized by effects of addition or omission of substances in the mixture of adhesion assay and enzymatic and chemical treatments of bacterial cells.

Effects of addition or omission (when stated) of substances to adhesion assay included omission of whey in NCTC 135 medium, fucose, glucose, galactose, or fructose (0.12 M); $CaCl_2$ (3.6 mM); and EDTA (4.5% wt/vol). Spent culture fluids were collected by centrifuging at 10,000 × *g* for 20 min and dialyzing (molecular weight 12 to 14 kDa) at least three times at 4°C for approximately 6 h against physiological saline instead of PBS.

All enzymes and chemicals were obtained from Sigma. Bacterial cells were washed once in PBS (pH 7.4) and then suspended in 0.5 ml of one of the following solutions: (i) periodate (0.05 M) in 0.1 M citrate-phosphate and 0.1 M NaCl buffer (pH 4.5) for 30 min at 37°C; (ii) iodate (0.05 M) in 0.1 M citrate-phosphate and 0.1 M NaCl buffer (pH 4.5) for 30 min at 37°C used as control; (iii) lipase (4 g/liter) in PBS for 1 h at 37°C; (iv) trypsin (5 g/liter) in 0.05 M Tris-HCl buffer (pH 8) for 1 h at 37°C; (v) protease from *Bacillus polymyxa* (Sigma) (0.2 g/liter) in PBS (pH 7.4) for 1 h at 37°C; or (vi) extraction of lectin with phenylmethylsulfonyl fluoride (0.03 M) in PBS containing 850 mM NaCl, twice homogenized for 30 min at 0°C. After each of these treatments, the bacteria were collected by centrifugation at 10,000 × *g* for 20 min and then washed twice in the same buffer.

Preparation for scanning electron microscopy. Cell pellets containing lactobacilli were prefixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) for 2 h. After fixation, the samples were washed three times with 0.1 M cacodylate buffer at 10-min intervals, and the samples were postfixed in 2% osmium tetroxide in 0.1 M cacodylate buffer for 2 h. Cells were then washed again three times using the same cacodylate buffer. Dehydration was carried out with ascending concentrations of acetone: 35, 50, 75, 95 (10 min each), and 100% (15 min; three times). The cells were then dried in a critical point drier (HCP-2, Hitachi, Tokyo, Japan) for 20 min and coated with gold using a scanning electron microscopy coating unit (E5100). The samples were examined with a scanning electron microscope (JEOL JSM 6400).

Statistical analysis. Experiments were carried out in triplicate. Significant differences were tested with HSO Tukey test by Minitab Statistic Program, release 8.21.

RESULTS

L. casei from human origin (CRL 431 and CRL 575) showed high adhesion ability to IECs, whereas *L. casei*

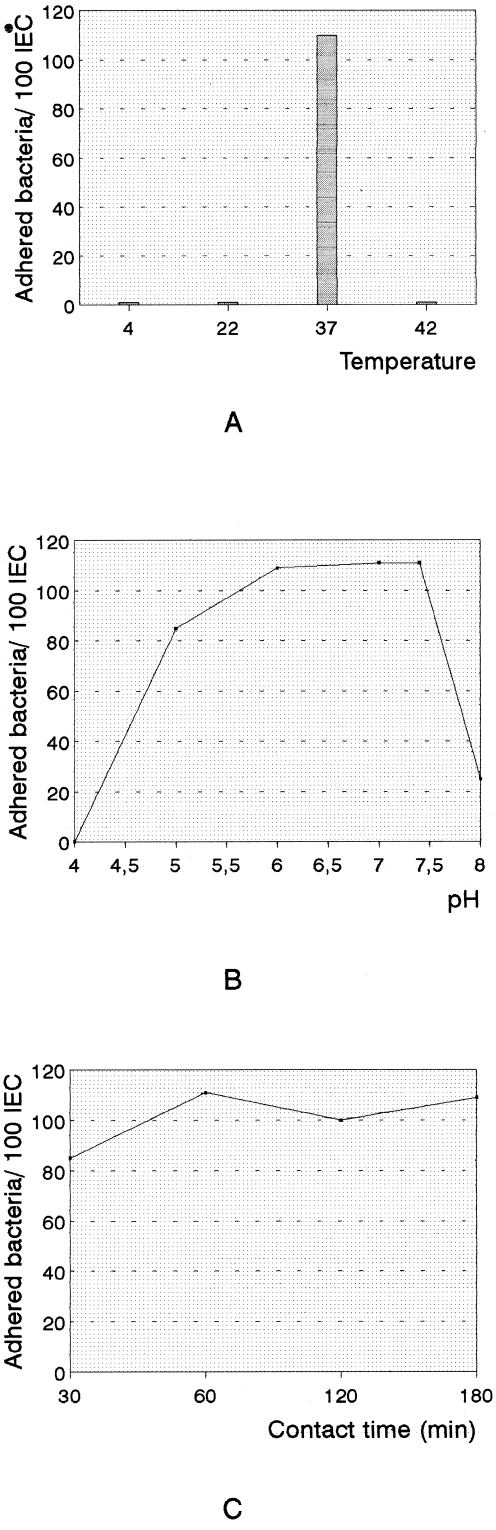


FIGURE 1. *Effects of incubation temperature (A), pH of buffer (B), and incubation time (C) on the attachment of* L. casei *CRL 431 to IECs of mice. Adhered bacteria are expressed per 100 IECs.*

CRL 55 from dairy origin and *L. acidophilus* CRL 730 from human origin showed low ability (Table 1). Variations in pH of the suspension buffers had significant effect on the attachment of *L. casei* CRL 431 to IECs. Highest values of attachment were obtained at a pH range from 6 to 7.5.

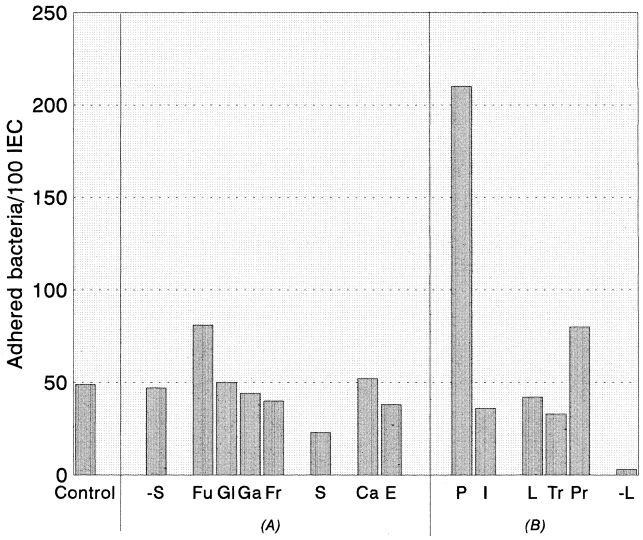
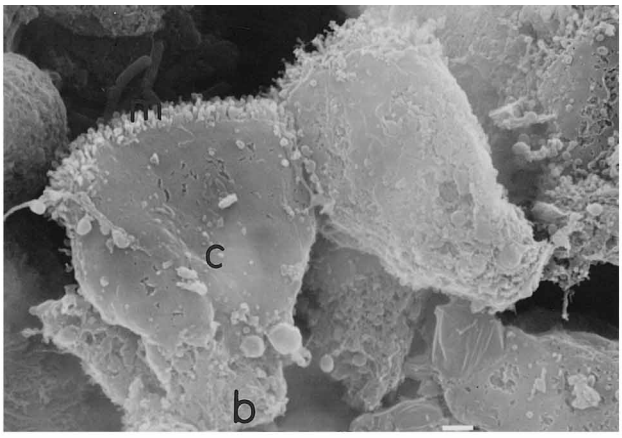


FIGURE 2. *(A) Effects of addition or exclusion of substances to or from the reaction mixture: exclusion of whey (−S) and addition of fucose (Fu), glucose (Gl), galactose (Ga), fructose (Fr), culture supernatant (S), calcium (Ca), and EDTA (E). (B) Effects of chemical and enzymatic treatments: iodate (I), periodate (P), lipase (L), trypsin (Tr), protease (Pr), and extraction of lectin (−L) on adherence of* L. casei *CRL 431 to IECs of mice. Adhered bacteria are expressed per 100 IECs.*

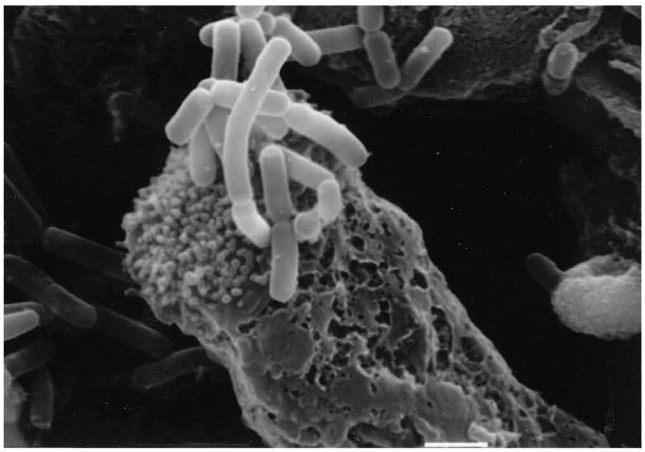
When the pH was lowered (less than 6.0), the adhesion gradually decreased, but when the pH was increased (higher than 7.5), the adhesion decreased abruptly (Fig. 1). No significant difference was observed in the number of bacteria attached to IECs after incubation times of 60, 120, and 180 min, but 30 min showed significantly lower values (Fig. 1). The adhesion phenomenon was sensitive to temperature changes, and *L. casei* CRL 431 was only able to attach to IECs at 37°C (Fig. 1).

Since fetal bovine serum usually interferes with adhesion, this was excluded from the culture medium, but no significant differences could be noticed with respect to adhesion (Fig. 2). Addition of fucose to the reaction mixture for attachment assays showed significant differences with respect to the control ($P < 0.01$). Addition of glucose, galactose, or fructose gave values similar to the control ones. The supernatant was able to inhibit adhesion to IECs and may contain cell wall fractions released during turnover. Addition of calcium and removal of divalent cations through EDTA and addition of calcium did not cause any significant variations with respect to controls (Fig. 2).

L. casei CRL 431 cells treated with periodate (oxidizing agent of cell surface carbohydrates) showed adhesion values four times higher than controls (Fig. 2). On the other hand, iodate, as a control for the effect of iodine in the previous assay, did not increase adhesion to IECs; a slight decrease was noticed. Cells treated with protease significantly increased adhesion to IECs with about 60%. Extraction of lectinlike substances from the surface of *L. casei* eliminated the ability of attachment to IECs. Lipase and trypsin did not produce any significant difference when compared with the controls (Fig. 2). Scanning electron microscopic images show that exfoliated IECs conserved



(A)



(B)

FIGURE 3. *Scanning electron micrograph of (A) IECs of mice without bacteria: microvilli (m), cell contact region (c), and cell base (b) (magnification ×6,000); (B) adhesion of* L. casei *CRL 431 to IECs of mice to the microvilli region (magnification ×12,000, bar = 1 μm)*

completeness and morphologic structure, which validates the extraction technique (Fig. 3A). The microvilli region, contact zone, and cell base can be clearly observed.

DISCUSSION

We have previously found a structural characteristic in *L. casei* CRL 431 with immunostimulating capacity: molecules emerged from the cell surface. These molecules were identified as lectinlike structures, and the immunostimulation observed can be started by an adhesion phenomenon in which these structures are involved *(15, 16).* This article shows that *L. casei* CRL 431 can attach to IECs from mice, and this adhesion could function in vivo as the initial step in the immunostimulation process. *L. acidophilus,* however, showed low adherence ability, which is in agreement with previous conclusions about its incompetence for phagocytic stimulation after oral administration *(16).*

The pH range with highest adhesion capacity was between 6 and 7.5, which is within the normal pH range of the intestinal environment (6 to 8) *(14).* Therefore, all subsequent experiments were performed at pH 7.4. The exposure time necessary to obtain highest adhesion values was superior to 30 min, which is in agreement with results obtained by Jin et al. *(13).* The contact time required for attachment reported by other authors varies from as short as a few minutes *(4)* to 1 h *(3).* Adhesion was only observed at 37°C. This would indicate that the biochemical and physical factors involved in adherence do not function at 22 or 42°C. However, Fuller *(8)* and Jin et al. *(13)* have reported that temperatures similar to those used in our work did not disturb adhesion.

Adhesion values for attachment of *L. casei* to IECs were improved by adding fucose in which the C_6 is deprived of an OH group. As a result, the C_6 region becomes nonpolar and hydrophobic. Fucose molecules have an amphipatic character. Our results could indicate that a mediator with similar nature to fucose is a necessary tool in the adhesion mechanism of *L. casei* CRL 431. This property can be important, considering that the intestinal mucin of rodents is rich in fucose *(21).* On the contrary, addition of neutralized supernatant of *L. casei* to the reaction mixture showed only half the adhesion value obtained from the control assay. This could be due to certain substances released during bacterial growth, which are able to influence the adhesion phenomenon as reported by Greene and Klaenhammer *(11).* The supernatant able to inhibit adhesion to IECs may contain cell wall fractions released during turnover. In previous studies *(16),* we have demonstrated that cell wall fractions obtained from *L. casei* CRL 431 produced immunostimulation in mice. If it is assumed that adhesion is the first step in immunostimulation, then it is feasible that IECs in the reaction mixture are partially occupied before addition of whole cells of *L. casei*.

Since fetal bovine serum usually interferes with adhesion, this was excluded from the culture medium, but no significant differences could be noticed with respect to adhesion. Removal (by EDTA) or addition of calcium did not cause any significant variations with respect to controls, unlike results observed by Conway and Kjelleberg *(5),* which suggested bivalent cations are required for adhesion.

Probably the oxidation process caused by periodate determines physical and chemical changes on the bacterial surface, which improve cell stability in suspension and could permit a low-specificity adhesion. In this case, bacteria that are able to adhere to IECs with low specificity probably released certain molecules, producing a particular state on cell surfaces. Cells treated with trypsin and lipase before testing showed levels of adhesion similar to the controls. The technique for extraction of lectins in presence of phenylmethylsulfonyl fluoride designed for *E. coli (6)* released lectinlike substances in *L. casei* CRL 431 *(15).* The substance responsible for attachment of *L. casei*

to IECs was removed by this treatment. These results agree with previous studies on immunostimulation after oral administration of cell wall fractions and peptidoglycan from *L. casei* CRL 431 *(16).* The ability of this strain to stimulate phagocytosis is lost after elimination of the lectinlike substance. From these results, it can be inferred that phagocytosis stimulation would be enhanced after binding closely to intestinal mucosa *(16).* In the present work, we confirm that this lectinlike structure is involved in the adhesion to IECs, because after removal adhesion values are negligible (Fig. 2). Our study shows that a strain with immunostimulating ability presents good adhesion qualities to IECs.

L. casei CRL 431 and *L. acidophilus* CRL 730 are used together in fermented milk with biotherapeutic properties *(9, 10). L. casei* was found to be the most effective strain with respect to immunostimulating capacity after oral administration as either whole cells or cell wall fractions *(16, 19). L. casei* CRL 431 adheres to the microvilli region. Cells do not adhere isolated but only after forming clusters (Fig. 3B), which seems to disagree with the data given in Table 1. Table 1 shows the results of the adhesion assay of the bacterial suspension with shaking, giving about one bacterium per cell. However, during preparation of the scanning electron microscopy sample agglutination occurs, because the substance is concentrated on a grill, which gives bacteria the opportunity to cluster. Adhesion in clusters has been previously observed in *L. acidophilus* BG2FO4 to HT-29 cells *(3).* Adhesion studies of *L. casei* and *L. acidophilus* to human intestinal cells obtained through exfoliation of samples extracted for biopsy are being developed.

ACKNOWLEDGMENTS

This work was supported financially by CIUNT-26 D-126. The authors thank LAMENOA (Electronic Microscopy Laboratory UNT) for technical assistance with the microscopy study.

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