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Lactobacillus casei DN-114 001 Inhibits the Ability of Adherent-Invasive *Escherichia coli* Isolated from Crohn's Disease Patients To Adhere to and To Invade Intestinal Epithelial Cells

Isabelle Ingrassia,[1] Antony Leplingard,[2] and Arlette Darfeuille-Michaud[1]*

Pathogénie Bactérienne Intestinale, CBRV, Université d'Auvergne, Clermont-Ferrand, France,[1] *and Danone Vitapole, Nutrivaleur, Palaiseau, France*[2]

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Ileal lesions in 36.4% of patients with Crohn's disease are colonized by pathogenic adherent-invasive *Escherichia coli*. The aim of this study was to determine the in vitro inhibitory effects of the probiotic strain, *Lactobacillus casei* DN-114 001, on adhesion to and invasion of human intestinal epithelial cells by adherent-invasive *E. coli* isolated from Crohn's disease patients. The experiments were performed with undifferentiated Intestine-407 cells and with undifferentiated or differentiated Caco-2 intestinal epithelial cells. Bacterial adhesion to and invasion of intestinal epithelial cells were assessed by counting CFU. The inhibitory effects of *L. casei* were determined after coincubation with adherent-invasive *E. coli* or after preincubation of intestinal cells with *L. casei* prior to infection with adherent-invasive *E. coli*. Inhibitory effects of *L. casei* on adherent-invasive *E. coli* adhesion to differentiated and undifferentiated intestinal epithelial cells reached 75% to 84% in coincubation and 43% to 62% in preincubation experiments, according to the cell lines used. Addition of *L. casei* culture supernatant to the incubation medium increased *L. casei* adhesion to intestinal epithelial cells and enhanced the inhibitory effects of *L. casei*. The inhibitory effects on *E. coli* invasion paralleled those on adhesion. This effect was not due to a bactericidal effect on adherent-invasive *E. coli* or to a cytotoxic effect on epithelial intestinal cells. As *Lactobacillus casei* DN-114 001 strongly inhibits interaction of adherent-invasive *E. coli* with intestinal epithelial cells, this finding suggests that the probiotic strain could be of therapeutic value in Crohn's disease.

Crohn's disease (CD) is a chronic inflammatory bowel disease (IBD) for which the etiology is still unknown, but several factors, including genetic, environmental, immunological, and other endogenous host factors, have been incriminated (40). Among the environmental triggers, luminal bacteria seem to play a substantial role. Indeed, the onset of inflammation in IBD may be associated with an imbalance in the intestinal microflora, with a relative predominance of aggressive bacteria and an insufficient amount of protective bacteria (46). Moreover, the efficacy of antibiotic therapy suggests a role of bacterial flora in CD. However, antibiotic treatments are often associated with gastrointestinal side effects and bacterial resistance, which may contribute to treatment failure (14, 21, 41, 43, 48).

In early and chronic ileal lesions of CD, an abnormal predominance of *Escherichia coli* has been observed (between 50 and 100% of the total number of aerobes and anaerobes). Most of these strains are able to adhere to and invade intestinal epithelial cells and to replicate within macrophages (9, 17, 19). These *E. coli* strains belong to a pathogenic group of *E. coli* designated AIEC (for adherent-invasive *E. coli*) (9). In neoterminal ileal specimens, AIEC strains were found in 36.4% of CD patients (16). Treatments aimed at eradicating these pathogenic strains and replacing them by nonpathogenic bacteria such as probiotic strains may be beneficial for the course of CD and may provide an innovative approach to treatment.

Probiotics are living microorganisms that upon ingestion in sufficient numbers exert benefits on human health. By modulating enteric flora, probiotic strains are effective in the prevention and treatment of antibiotic-associated, rotavirus, *Clostridium difficile*-associated, or traveler's diarrhea (for a review, see reference 47). The efficacy of several probiotics for IBD has been investigated in clinical trials. *E. coli* Nissle 1917, *Saccharomyces boulardii*, and a formula consisting of species of *Bifidobacterium*, *Lactobacillus*, and *Streptococcus salivarius* subsp. Thermophilus (VSL #3) have been reported as being as effective as standard treatment in preventing relapse in ulcerative colitis and chronic pouchitis (24, 42, 51). Probiotics are used in these pathologies basically to restore the unbalanced indigenous microflora, to inhibit the adverse effects of enteric pathogens, and to counteract the inflammatory process (28, 45).

Lactobacillus casei DN-114 001 is a probiotic strain that survives intestinal transit (35) and exerts beneficial effects in vivo. It is able to modify the digestive microflora and enhance the immune system during its transit in the digestive tract (23, 39). It was shown to reduce the incidence and duration of diarrhea in children (37, 38). Moreover, a recent study has provided evidence that this probiotic interacts with human intestinal mucosa and can markedly reduced the mucosal re-

* Corresponding author. Mailing address: Pathogénie Bactérienne Intestinale, Laboratoire de Bactériologie, CBRV, 28 Place Henri Dunant, 63000 Clermont-Ferrand, France. Phone: 33 4 73 17 79 97. Fax: 33 4 73 17 83 71. E-mail: arlette.darfeuille-michaud@u-clermont1.fr.

lease of tumor necrosis factor alpha and interleukin-8 in active Crohn's disease (5, 6).

The aim of the present study was to investigate whether *L. casei* DN-114 001 could inhibit the ability of pathogenic adherent-invasive-*E. coli* strains isolated from patients with Crohn's disease to adhere to and invade intestinal epithelial cells in vitro.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *L. casei* DN-114 001 was provided by Danone Vitapole (Paris, France). *L. casei* DN-114 001 was grown in De Man, Rogosa, and Sharpe (MRS) broth (Difco, Becton Dickinson, Meylan, France) at 37°C for 18 h. The culture was centrifuged (10,000 × *g* for 5 min at 4°C), and bacteria were suspended in cell culture medium. The final suspension was adjusted to obtain the appropriate concentration. The number of CFU was determined by plating serial 10-fold dilutions from bacterial suspensions on MRS agar plates. Plates were incubated at 37°C in a CO_2 atmosphere for 48 h.

Seven AIEC strains were assessed: the AIEC reference strain LF82 (9) and strains LF9, LF15, LF31, LF65, LF110, and LF134 (16). All these strains isolated from patients with Crohn's disease were characterized by using the adhesion and invasion assays described below. All strains were highly sensitive to gentamicin. *E. coli* LF32 was used as a positive control for cytotoxicity assay, since this strain produces α-hemolysin (17). *E. coli* strain K-12 C600 was used as a negative control. All *E. coli* strains were grown either in Luria-Bertani broth without shaking or on Mueller-Hinton agar plates (Institut Pasteur Production, Marnes-la-Coquette, France) overnight at 37°C.

Intestinal cell lines and cell cultures. The Intestine-407 cells (ATCC CCL6; Flow Laboratories, Inc., McLean, VA) derived from human embryonic jejunum and ileum were used as an intestinal model for undifferentiated intestinal epithelial cells mimicking the cells found in the crypts of the intestinal villi. They were cultured for 20 h and in an atmosphere containing 5% CO_2 at 37°C in Eagle minimum essential medium (Eagle MEM; BioWhittaker-Cambrex, Emerainville, France) supplemented with 10% (vol/vol) fetal bovine serum (BioWhittaker-Cambrex, Emerainville, France), 1% (vol/vol) nonessential amino acids (BioWhittaker), 1% (vol/vol) L-glutamine (Gibco BRL-Life Technologies, Cergy-Pontoise, France), 200 U of penicillin, 50 mg of streptomycin, 0.25 mg/liter of amphotericin B (Gibco BRL-Life Technologies, Cergy-Pontoise, France), and 1% (vol/vol) MEM vitamin solution X-100 (BioWhittaker). Caco-2 cells established from human colonic adenocarcinoma were kindly provided by Alain Zweibaum (INSERM U178, Villejuif, France). These cells were used as undifferentiated cells to mimic cells of the crypts and as differentiated cells to mimic mature enterocytes of the small intestine (30). Undifferentiated and differentiated Caco-2 cells were grown for 2 days and 15 days, respectively. Cells were cultured in Dulbecco's modified Eagle medium (DMEM) with 4.5 g/liter of glucose (BioWhittaker) supplemented with 20% (vol/vol) fetal bovine serum (BioWhittaker), 1% (vol/vol) nonessential amino acids (BioWhittaker), 1% (vol/vol) L-glutamine (Gibco BRL-Life Technologies, Cergy-Pontoise, France), 200 U of penicillin, 50 mg of streptomycin, 0.25 mg/liter of amphotericin B (Gibco BRL-Life Technologies, Cergy-Pontoise, France), and 1% (vol/vol) MEM vitamin solution X-100 (BioWhittaker). The cells were grown at 37°C in 5% CO_2.

Adhesion and invasion assays. Intestine-407 cells were seeded in 24-well tissue culture plates (Polylabo, Strasbourg, France) at 4×10^5 cells per well and grown for 20 h. Caco-2 cells were seeded in 24-well tissue culture plates (Polylabo, Strasbourg, France) at 2×10^5 cells per well and grown for 2 days for undifferentiated cells and 15 days for differentiated cells. The culture medium was changed every 2 days. The cells were washed twice with phosphate-buffered saline (BioWhittaker).

To study the adhesion of *L. casei* DN-114 001, each cell line was infected in 1 ml of the cell culture medium supplemented with heat-inactivated (30 min; 56°C) fetal bovine serum, at a multiplicity of infection (MOI) of either 10, 100, or 500 bacteria per epithelial cell. After a 1- to 6-h incubation period at 37°C with 10% CO_2, the infected cells were washed three times with phosphate-buffered saline. To determine the total number of cell-associated bacteria, the cells were lysed with 1% (vol/vol) Triton X-100 (Sigma) in deionized water. This concentration of Triton X-100 did not affect bacterial viability for at least 30 min (data not shown). Samples were diluted and plated onto MRS agar plates to determine the number of CFU recovered from the lysed cells. To study the effect of spent *L. casei* culture supernatant (SN) on the adhesion of the strain, 10% (vol/vol) of its spent culture SN or neutralized spent culture SN were added to the cell culture medium. The spent culture supernatant of *L. casei* DN-114 001 was centrifuged and sterilized by filtration through a sterile 0.22-μm-pore-size filter unit (Millipore Molsheim, France). To check for potential interference of pH reduction linked to organic acid production, the spent culture supernatant was also adjusted to a neutral value (pH 7.0) using 4 M NaOH.

AIEC adhesion was measured utilizing the same protocol. MOIs of 10 and 100 were used. To determine the number of CFU recovered from the lysed cells, samples were diluted and plated onto Mueller-Hinton agar plates. For invasion assays, fresh cell culture medium containing 100 μg/ml of gentamicin was added after the infection period to kill extracellular bacteria. After incubation for an additional hour, cultured cells were treated as described above. Each assay was performed three times with successive passages of intestinal cells.

Adhesion and invasion inhibition assays. Two different procedures were used to assess exclusion of AIEC strain by *L. casei* DN-114 001 and competition between the two strains. Exclusion was assessed by performing preinfection experiments in which cultured intestinal epithelial cells were first incubated with *L. casei* DN-114 001 (MOI, 500) alone or in the presence of 10% of its spent culture supernatant for 6 h at 37°C. AIEC strain LF82 (MOI, 100) was added and incubation was continued for a further 3 h. Competition was assessed by performing coinfection experiments in which *L. casei* DN-114 001 (MOI, 500) bacteria alone or in the presence of 10% of its spent culture supernatant (or neutralized spent culture supernatant), and each of the AIEC strains tested were added to the cultured cells at an MOI of 10 for 6 h. The numbers of strains adhering to or invading the intestinal cells were determined as described above. For each assay, a minimum of three experiments was performed with successive passage of intestinal cells. To evaluate the number of adherent or intracellular bacteria per intestinal epithelial cells, two additional wells were prepared when the cells were seeded. At the end of the culture period, the cells were trypsinized and enumerated microscopically.

Epithelial cell viability: lactate dehydrogenase (LDH) measurement. At the same time as each study of *L. casei* DN-114 001 adherence, a duplicate 24-well plate of cultured epithelial cells was inoculated with bacteria and assayed as described above. At the end of the incubation period, supernatants of the infected cells containing released LDH were collected, centrifuged at 2,500 × *g* for 3 min at 4°C, and assayed for lactate dehydrogenase activity. Enzymatic activity was determined in the supernatants by using NADH as the substrate. Release of LDH was expressed as units per liter of supernatant. The percentage of cytotoxicity was calculated as follows: [(experimental release − spontaneous release)/(total release − spontaneous release)] × 100, where spontaneous release is the amount of LDH activity in supernatants of cells incubated in medium alone and total release is the LDH activity measured in cell lysates. *E. coli* LF32 producing an α-hemolysin was used as a positive control (17). *E. coli* strain K-12 C600 was used as a negative control.

Statistical analysis. The data were analyzed by Student's *t* test. *P* values of ≤0.05 were considered to be statistically significant.

RESULTS

Ability of *L. casei* DN-114 001 to adhere to intestinal epithelial cells. The ability of *L. casei* DN-114 001 to adhere to intestinal epithelial cells was determined using undifferentiated Intestine-407 and Caco-2 cells and differentiated Caco-2 cells. The adhesion of *L. casei* DN-114 001 increased with the MOI as shown in Fig. 1A. It also increased with the incubation period (Fig. 1B). The observed levels of adhesion varied according to the cell lines tested. With Intestine-407 cells, adhesion was maximal after 3 h of incubation, reaching an adhesion level of 3 bacteria/cell. With undifferentiated and differentiated Caco-2 cells, marked increases in adhesion levels were observed between 3 and 6 h of incubation. After 6 h of incubation, the adhesion levels were 2.7 and 3.9 bacteria/cell with undifferentiated and differentiated Caco-2 cells, respectively. Thus, *L. casei* DN-114 001 exhibited a dose-dependent and incubation time-dependent ability to adhere to undifferentiated and differentiated intestinal epithelial cells.

Increased ability of *L. casei* DN-114 001 to adhere to intestinal epithelial cells in the presence of its spent culture supernatant. Since *Lactobacilli* can produce secreted compounds able to interact with their adhesive abilities (10, 22), the effects

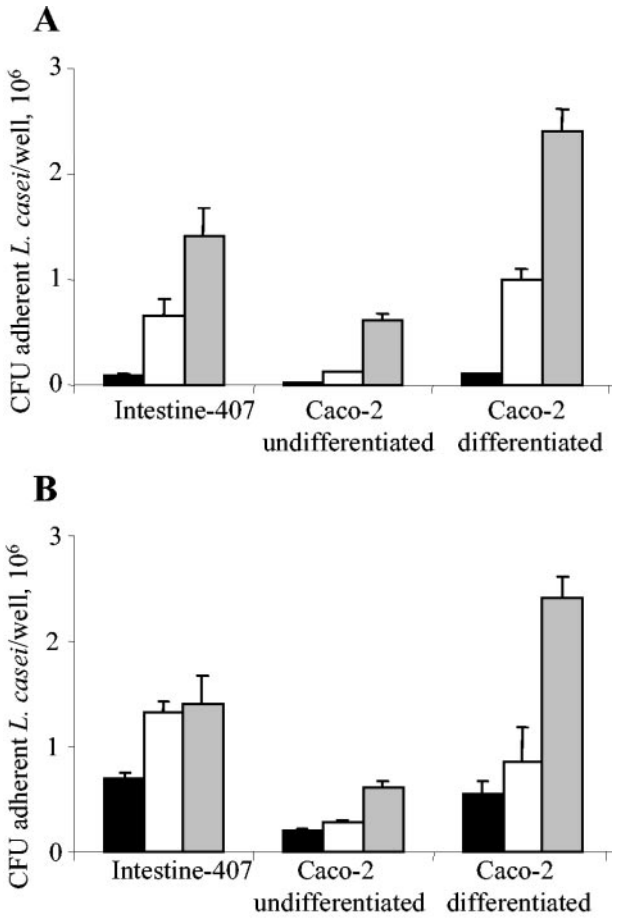

FIG. 1. Adhesion of *L. casei* DN-114 001 to intestinal epithelial cells according to multiplicity of infection (A) or to time of incubation (B). Adhesion of *L. casei* DN-114 001 was tested with undifferentiated Intestine-407 or Caco-2 cells cultured for 2 days and with differentiated Caco-2 cells cultured for 15 days. (A) Cultured cells were incubated at an MOI of 10 (black bars), 100 (white bars), and 500 (grey bars) for 6 h. (B) Cultured cells were incubated at an MOI of 500 for 1 h (black bars), 3 h (white bars), and 6 h (grey bars). Adhesion levels are expressed as the number of CFU per well. Data are given as means ± the standard error of the mean (SEM) of at least three separate experiments.

of putative secreted products on ability to adhere were tested. *L. casei* DN-114 001 adhesion assays were performed at an MOI of 500 for 6 h in the presence of 10% of *L. casei* DN-114 001 spent culture supernatant. The addition of 10% of *L. casei* DN-114 001 spent culture supernatant to the incubation medium induced 6.8-, 7.7-, and 7.1-fold increases in levels of adhesion of *L. casei* DN-114 001 to Intestine-407, undifferentiated Caco-2, and differentiated Caco-2 cells, respectively (Fig. 2). Since the observed effect could be related to a drop in pH due to acid lactic production, we performed similar experiments in the presence of 10% spent culture supernatant adjusted to a neutral pH value. It continued to induce a marked increase in *L. casei* DN-114 001 adhesion levels, indicating that an acidic pH was not the main factor involved in the increased ability of *L. casei* DN-114 001 to adhere to undifferentiated and differentiated intestinal epithelial cells.

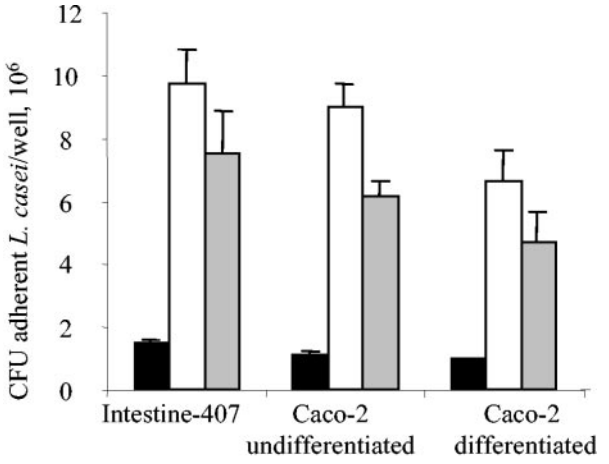

FIG. 2. Increased adhesion of *L. casei* DN-114 001 in the presence of its spent culture supernatant. Cultured cells were incubated with *L. casei* DN-114 001 at an MOI of 500 for 6 h in cell culture medium alone (black bars), or supplemented with 10% (vol/vol) of its spent culture supernatant (white bars) and with 10% of neutralized supernatant (grey bars). Adhesion levels were determined as described in the legend to Fig. 1.

Inhibitory effect of *L. casei DN* 114 001 on AIEC LF82 adhesion and invasion in preincubation experiments. The probiotic activity of *L. casei* DN-114 001 in terms of antiadhesive and anti-invasive effects on adherent-invasive *E. coli* colonization of the gut was determined in vitro using undifferentiated and differentiated intestinal epithelial cells.

Preincubation of cultured intestinal epithelial cells was performed with *L. casei* DN-114 001 prior to infection with AIEC LF82. As shown in Fig. 3A, *L. casei* DN-114 001 significantly ($P < 0.05$) inhibited the ability of LF82 to adhere to undifferentiated Intestine-407 cells (62%) and to undifferentiated and differentiated Caco-2 cells (47% and 43%, respectively). The inhibitory effects on LF82 adhesion were significantly ($P < 0.01$) increased when the preincubation of *L. casei* DN-114 001 was performed in the presence of 10% of its spent culture supernatant. Under such conditions, the LF82 adhesion level to Intestine-407 cells was reduced by 96%, and we observed percentages of inhibition of 73% and 70% on LF82 adhesion to undifferentiated and differentiated Caco-2 cells, respectively.

The inhibitory effect of *L. casei* DN-114 001 on LF82 invasion was only examined with undifferentiated intestinal epithelial cells (Intestine-407 and Caco-2 cells), since low levels of intracellular LF82 are observed with differentiated intestinal cells (9). The inhibitory effects of *L. casei* DN-114 001 on LF82 invasion were slightly higher than those obtained on LF82 adhesion (Fig. 3B). In preincubation experiments of intestinal epithelial cells with *L. casei* DN-114 001 alone, inhibitory effects on LF82 invasion of 90% with Intestine-407 and 56% with Caco-2 cells were observed. When preincubation with *L. casei* DN-114 001 was performed in the presence of 10% of its spent culture supernatant, inhibition of LF82 invasion was 98.7% with Intestine-407 and 89% with Caco-2 cells.

Inhibitory effect of *L. casei DN* 114 001 on AIEC adhesion and invasion in coincubation experiments. Adhesion and invasion levels of AIEC strains with respect to intestinal epithe-

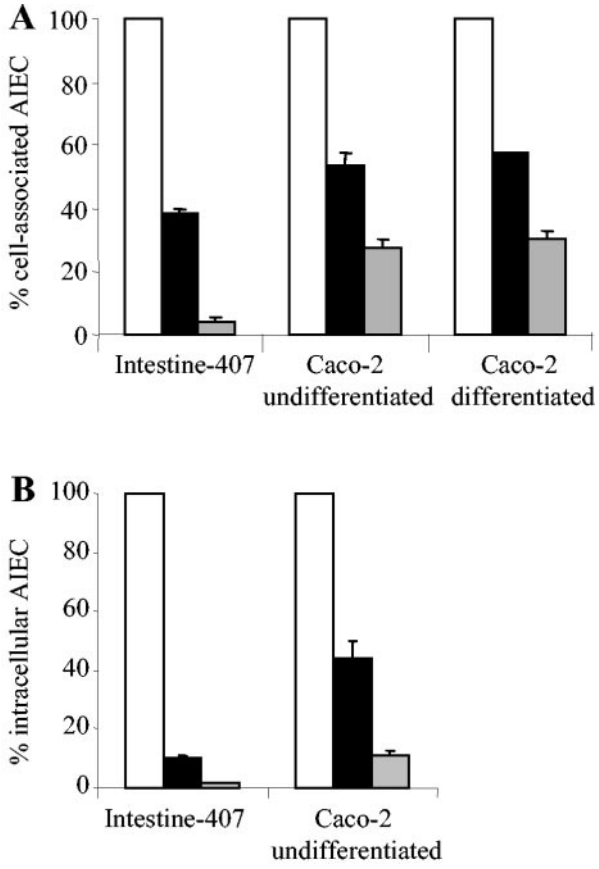

FIG. 3. Inhibitory effects of *L. casei* DN-114 001 on the abilities of AIEC LF82 to adhere to and to invade intestinal epithelial cells in preincubation experiments. Adhesion (A) and invasion (B) of AIEC LF82 with intestinal epithelial cells preincubated with *L. casei* DN-114 001 alone (black bars) or supplemented with 10% (vol/vol) of its spent culture supernatant (grey bars), compared with adhesion and invasion levels of AIEC LF82 to untreated epithelial cells (white bars), taken as 100%. Preincubation of cultured cells was performed for 6 h with *L. casei* DN-114 001 at an MOI of 500. Infection with AIEC LF82 was performed for 3 h with an MOI of 100. Invasion was determined after gentamicin treatment for an additional hour. Results are expressed as cell-associated bacteria (adherent plus intracellular bacteria) or intracellular bacteria relative to those obtained for strain LF82 with untreated cells. Each value is the mean ± SEM of three to four separate experiments.

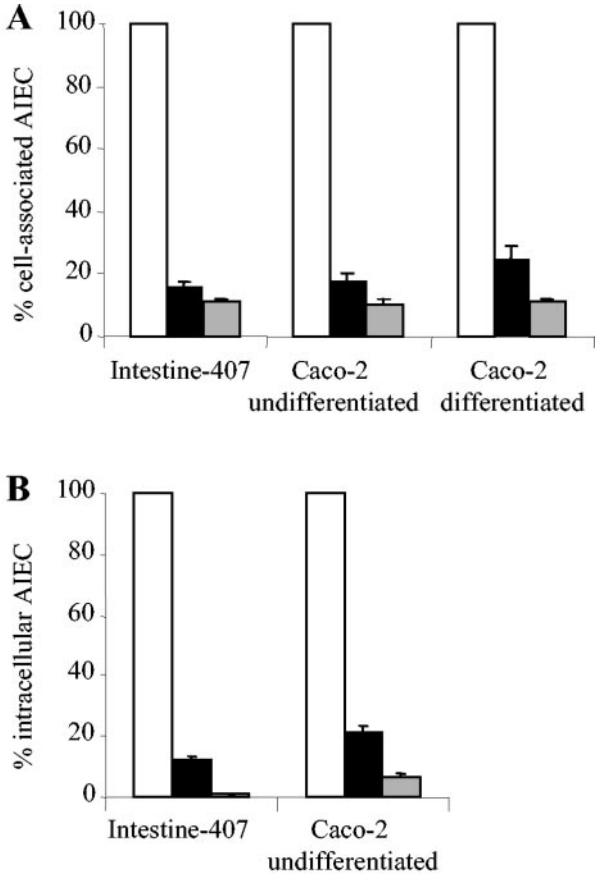

FIG. 4. Adhesion (A) and invasion (B) abilities of AIEC LF82 with respect to intestinal epithelial cells in coincubation experiments with *L. casei* DN-114 001 alone (black bars) or supplemented with 10% (vol/vol) of *L. casei* DN-114 001 spent culture supernatant (grey bars), compared with monoinfection experiments with LF82 alone (white bars). A multiplicity of infection of 500 was used for *L. casei* DN-114 001 and an MOI of 10 was used for AIEC LF82. Cell-associated bacteria were quantified after a 6-h incubation period. Invasion was determined after gentamicin treatment for an additional hour. Results are expressed as the percentage of cell-associated bacteria (adherent plus intracellular bacteria) or intracellular bacteria relative to those obtained in monoinfection with strain LF82, taken as 100%. Each value is the mean ± SEM of three to five separate experiments.

lial cells were determined by coincubation experiments where AIEC and *L. casei* DN-114 001 were added together with intestinal epithelial cells. In coincubation with *L. casei* DN-114 001, very marked decreases in AIEC LF82 adhesion levels were observed (Fig. 4A) with differentiated and undifferentiated cells. The inhibitory effect on LF82 adhesion was highly significant ($P < 0.01$) and percentage of inhibition was 82% and 84% with undifferentiated Caco-2 and Intestine-407 cells, respectively. It was 75% with differentiated Caco-2 cells. The inhibitory effect was even more pronounced when coincubations of AIEC LF82 and *L. casei* DN-114 001 were performed in the presence of 10% of *L. casei* DN-114 001 spent culture supernatant. Under these conditions, shown above to increase the ability of *L. casei* DN-114 001 to adhere to intestinal epithelial cells, the percentage of inhibition of LF82 adhesion was 89, 90, and 89% with Intestine-407, undifferentiated Caco-2, and differentiated Caco-2 cells, respectively. When coincubation of AIEC LF82 and *L. casei* DN-114 001 was performed in the presence of 10% of neutralized *L. casei* spent culture supernatant, similar inhibitory effects on AIEC LF82 adhesion to Intestine-407 cells were observed (Table 1).

The inhibitory effect of *L. casei* DN-114 001 on LF82 invasion paralleled inhibition of adhesion (Fig. 4B). In coincubation with *L. casei* DN-114 001, the number of intracellular LF82 bacteria significantly ($P < 0.001$) decreased. The percentage of inhibition of LF82 invasion was similar to that observed with LF82 adhesion, 88 and 79% with Intestine-407 and Caco-2 cells, respectively. Similar to results obtained on LF82 adhesion, the addition of 10% of *L. casei* DN-114 001 spent culture supernatant in the incubation medium induced a more pronounced inhibitory effect on LF82 invasion, 99 and 93% with Intestine-407 and Caco-2 cells, respectively. When the *L. casei* culture supernatant was neutralized, similar inhib-

TABLE 1. Inhibition of adhesion and invasion of various AIEC strains by probiotic *L. casei* DN-114 001[c]

AIEC strain	Coincubation of *L. casei* DN-114 001 with:			
	10% *L. casei* spent culture SN		10% *L. casei* neutralized spent culture SN	
	% Inhibition of cell association[a]	% Inhibition of cell invasion[b]	% Inhibition of cell association[a]	% Inhibition of cell invasion[b]
LF 82	89.1 ± 2.8	99.7 ± 0.2	84.9 ± 4.1	99.4 ± 0.2
LF 9	87.1 ± 2.4	97.6 ± 0.7	80.3 ± 5.1	97.4 ± 0.7
LF 15	87.4 ± 2.8	98.0 ± 1.0	81.4 ± 3.6	97.8 ± 1.0
LF 31	90.0 ± 3.3	98.9 ± 0.6	86.9 ± 2.9	98.5 ± 0.6
LF 65	87.2 ± 3.4	98.9 ± 0.7	78.6 ± 3.9	98.6 ± 0.7
LF 110	89.1 ± 2.4	99.0 ± 0.6	86.0 ± 2.7	98.4 ± 0.8
LF 134	83.4 ± 2.7	96.1 ± 1.4	76.3 ± 4.0	93.0 ± 2.6

[a] Inhibition (%) = [(cell-associated bacteria alone − cell-associated bacteria in coincubation experiments with *L. casei* DN-114 001)/(cell-associated bacteria alone)] × 100.
[b] Inhibition (%) = [(intracellular bacteria alone − intracellular bacteria in coincubation experiments with *L. casei* DN-114 001)/(intracellular bacteria alone)] × 100.
[c] Coincubation experiments were performed with Intestine-407 cells for 6 h at a multiplicity of infection of 500 for the probiotic strain *L. casei* DN-114 001 and of 10 for AIEC strains. Results are expressed as a percent inhibition of cell-associated bacteria or intracellular bacteria relative to those obtained in monoinfections with AIEC strains (taken as 100%). Each value is the mean ± SEM of three separate experiments.

itory effects on AIEC LF82 invasion of Intestine-407 cells were observed (Table 1).

Inhibitory effects of *L. casei* DN-114 001 on bacterial adhesion and invasion of other AIEC strains were investigated with Intestine-407 cultured cells (Table 1). Strong inhibitory effects on AIEC adhesion and invasion were observed for all the AIEC strains tested. Adhesion inhibition levels ranged from 83 to 90%, and invasion inhibition levels ranged from 96 to 99%. These inhibitory effects continued to be observed when the pH of *L. casei* DN-114 001 spent culture supernatant was adjusted to a neutral value. Slight decreases were observed, but adhesion inhibition levels still ranged from 76 to 86%, and invasion inhibition levels ranged from 93 to 98%.

The inhibitory effect of *L. casei DN* 114 001 on AIEC LF82 adhesion and invasion is not related to cell cytotoxicity or to antibacterial activity. Since the decreased ability of AIEC LF82 to adhere to and invade intestinal epithelial cells may be related to cultured cell death induced by *L. casei* and/or its spent culture supernatant, we determined the amounts of the cytoplasmic enzyme LDH released when the integrity of the cytoplasmic membrane of eukaryotic cells was breached (Table 2). As a positive control inducing LDH release, we used *E. coli* strain LF32 producing an α-hemolysin (17). Infection of the different cell models with *E. coli* LF32 for 6 h induced marked release of LDH (19.3 to 22.7%). In contrast, LDH release by intestinal epithelial cells incubated for 6 h with an MOI of 500 of *L. casei* DN-114 001 alone or in the presence of 10% of its spent culture supernatant was very low (2.0 to 5.7), and values were similar to those observed when incubations were performed with the nonpathogenic strain *E. coli* K-12 C600. This indicates that *L. casei* DN-114 001 alone or in association with its spent culture supernatant did not induce any cell cytotoxicity even at an MOI of 500 and after 6 h of incubation with intestinal epithelial cells.

Since inhibition of LF82 adhesion and invasion may be related to bactericidal effects or inhibition of bacterial growth induced by *L. casei* DN-114 001, LF82 bacterial growth was analyzed by incubation with DMEM with *L. casei* DN-114 001 alone and in the presence of its spent culture supernatant (Table 3). Coincubation for 3 h with *L. casei* DN-114 001 alone or with 10% of its spent culture supernatant did not induce any significant ($P > 0.05$) decrease in LF82 bacterial growth. After 6 h of coincubation with *L. casei* DN-114 001, a 26% decrease in LF82 replication was observed. This decrease reached 38%

TABLE 2. Absence of LDH release by intestinal epithelial cells incubated with *L. casei* DN-114 001 alone and in the presence of its spent culture SN

Strain	% LDH release[a]		
	Intestine-407	Caco-2	
		Undifferentiated	Differentiated
E. coli LF 32	22.7 ± 0.2	19.3 ± 0.7	20.2 ± 2.0
E. coli K12-C600	2.6 ± 0.9	5.0 ± 0.4	6.2 ± 0.2
L. casei DN-114 001	2.0 ± 0.6	5.7 ± 0.7	2.3 ± 0.1
L. casei DN-114 001 + 10% SN	3.1 ± 0.9	5.2 ± 0.4	4.2 ± 0.9

[a] LDH release was determined in supernatants using NADH as the substrate. It was measured after 6 h of incubation with a multiplicity of infection of 500 for *L. casei* DN-114 001 and of 100 for *E. coli* strains. Results are expressed as percent cytotoxycity, calculated as (experimental release − spontaneous LDH release)/(total LDH release − spontaneous release) × 100. *E. coli* LF32 producing an α-hemolysin was used as a positive control (17). *E. coli* strain K-12 C600 was used as a negative control.

TABLE 3. Activity of *L. casei* DN-114 001 alone or in the presence of 10% of its spent culture supernatant on AIEC LF82 bacterial growth[a]

Growth medium	Bacterial growth in CFU (10^7)/ml (% of growth inhibition/DMEM)	
	Time of incubation	
	3 h	6 h
DMEM	2.9 ± 0.2	43.5 ± 5.2
DMEM + *L. casei* DN-114 001	3.0 ± 0.4 (0)	32.2 ± 3.3 (26)
DMEM + *L. casei* DN-114 001 + 10% SN	2.7 ± 0.4 (5)	26.7 ± 2.1 (38)
DMEM + *L. casei* DN-114 001 + 10% SN (pH = 7)	3.0 ± 0.3 (0)	31.8 ± 2.1 (27)

[a] Control bacterial growth of LF82 was determined in cell culture medium (DMEM). Bacterial growth of LF82 was also determined in coincubation with *L. casei* DN-114 001 alone or supplemented with 10% of *L. casei* DN-114 001 spent culture supernatant (10% SN) or of neutralized supernatant (10% SN, pH 7.0). Results are expressed as (CFU) after 3 h or 6 h incubation. Each value is the mean ± SEM of five separate experiments.

when coincubation of AIEC LF82 was performed with *L. casei* DN-114 001 in the presence of 10% of the spent supernatant. When the pH of the *L. casei* DN-114 001 supernatant was adjusted to a neutral value (pH 7.0), the decrease in LF82 bacterial growth was similar to that observed with *L. casei* alone. Compared to the inhibitory effects of *L. casei* on AIEC LF82 adhesion and invasion (89 to 98% of inhibition), the influence of *L. casei* on LF82 bacterial growth could not fully explain the resulting inhibitory effects of *L. casei* DN-114 001 on the abilities of strain LF82 to adhere to and invade cultured intestinal epithelial cells.

DISCUSSION

The onset of inflammation in IBD may be associated with an imbalance in the intestinal microflora, with a relative predominance of aggressive bacteria and an insufficient amount of protective species. Since early and chronic ileal lesions of patients with Crohn's disease are abnormally colonized by AIEC strains (16), the eradication of these pathogenic bacteria and their replacement by probiotic bacteria may provide a new option for the treatment of CD and for the maintenance of remission.

To provide clinical benefits, adhesion of probiotic bacteria to the intestinal mucosa is an interesting trait for antagonistic activity against pathogens. We showed in the present study that the probiotic strain *L. casei* DN-114 001 exhibited a dose- and incubation time-dependent ability to adhere to undifferentiated Intestine-407 and Caco-2 cells and to differentiated Caco-2 cells. This result was expected, since several studies showed that various *Lactobacillus* strains adhere to cultured intestinal epithelial cells (18, 20, 22, 29, 44, 50). Their ability to adhere is independent of bacterial species, but is strain specific (10, 49), and is increased in the presence of spent culture supernatant (3, 10, 12, 44, 50). A markedly increased ability of *L. casei* DN-114 001 to adhere to intestinal epithelial cells was also observed in the presence of 10% of its spent culture supernatant. This indicates that a secreted factor enhancing bacterial adhesion or the low pH due to acid lactic production may interfere with the adhesion of *Lactobacilli* to intestinal epithelial cells (3, 10, 12, 15, 22, 25). However, this study shows that low pH is not required for adhesion of *L. casei* DN-114 001 strain to adhere. Besides, Greene and Klaenhammer (22) previously reported that lowering the pH of fresh MRS cannot account for the increased adherence of *Lactobacilli* to Caco-2 cells following the addition of the spent culture supernatant.

An important function of probiotic bacteria is to provide protection of the host gastrointestinal tract from invading pathogens. In this study, we showed that preincubation of intestinal epithelial cells with *L. casei* DN-114 001 prior to infection with pathogenic AIEC strain LF82 resulted in a strong decrease in LF82 adhesion (43 to 62%) and invasion (56 to 90%). In coincubation experiments, where *L. casei* DN-114 001 and AIEC strain LF82 were added together with the intestinal epithelial cells, higher inhibitory effects on AIEC adhesion (75 to 84%) and invasion (79 to 88%) were observed. The inhibitory effects of *L. casei* DN-114 001 on both LF82 adhesion and invasion are not surprising, since this result is consistent with previous observations showing that the adhesion step is crucial for AIEC to invade epithelial cells (7). Several reports have confirmed the ability of probiotic *Lactobacilli* to inhibit the ability of pathogenic bacteria to adhere to and to invade intestinal epithelial cells (4, 10, 20, 26, 27). The inhibition levels observed with *L. casei* DN-114 001 are similar to or even higher than those reported for *Lactobacilli* on the adhesion of various pathogens (4, 11, 18, 20, 49). We noticed that *L. casei* DN-114 001 exerts stronger inhibitory effects on AIEC invasion than those reported with various *Lactobacilli* on invasive enteropathogens (11, 13, 20). Concerning its inhibitory role on AIEC adhesion and invasion, *L. casei* DN-114 001 is as efficient as *E. coli* Nissle (8).

The inhibitory activity of *L. casei* DN-114 001 on LF82 adhesion and invasion was enhanced when 10% of spent *L. casei* DN-114 001 culture supernatant was added to coincubation and preincubation media. This finding continued to be observed when the acidic pH of the supernatant was neutralized. The increased inhibitory effects may be related to the increased adhesion of *L. casei* DN-114 001 that we observed in the presence of its spent culture supernatant. Such increased adhesion could induce greater competition between probiotic and pathogens for attachment sites by specific blockage or steric hindrance. This could also be due to the production of antimicrobial substances active against AIEC. Several studies have reported the ability of *Lactobacilli* to secrete antimicrobial compounds such as bacteriocins (1, 2, 31, 36, 52, 53). Lactic acid produced by all *Lactobacillus* strains was also shown to inhibit bacterial growth (32–34). Under the experimental conditions of this study, even though the secretion by *L. casei* DN-114 001 of an antibacterial substance active on AIEC LF82 bacterial growth was observed, this substance alone could not explain the high inhibitory effects of *L. casei* DN-114 001 on the abilities of strain LF82 to adhere to and to invade cultured intestinal epithelial cells. Thus, the high inhibitory effects of *L. casei* on AIEC adhesion to and invasion of intestinal epithelial cells would result from several cumulative factors such as the adhesion of *L. casei* to the intestinal epithelial cells and the secretion by *L. casei* of compounds, which induce a high increase in *L. casei* adhesion and a moderate decrease in AIEC bacterial growth.

In conclusion, probiotic strain *L. casei* DN-114 001 exerts strong inhibitory effects on both AIEC adhesion to and invasion of intestinal epithelial cells. The present in vitro study indicates that this probiotic may be efficient for preventive and curative probiotic therapy, since inhibitory effects were observed when intestinal cells were preincubated with *L. casei* DN-114 001 and when the probiotic was used in coincubation experiments. Thus, the use of this probiotic could be of great interest, especially in maintaining remission in a subset of CD patients harboring pathogenic AIEC colonizing early and chronic ileal lesions.

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