Article

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BERNET, Marie-Françoise, *et al*.

Abstract

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Adhesion of Human Bifidobacterial Strains to Cultured Human Intestinal Epithelial Cells and Inhibition of Enteropathogen-Cell Interactions

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Thirteen human bifidobacterial strains were tested for their abilities to adhere to human enterocyte-like Caco-2 cells in culture. The adhering strains were also tested for binding to the mucus produced by the human mucus-secreting HT29-MTX cell line in culture. A high level of calcium-independent adherence was observed for *Bifidobacterium breve* 4, for *Bifidobacterium infantis* 1, and for three fresh human isolates from adults. As observed by scanning electron microscopy, adhesion occurs to the apical brush border of the enterocytic Caco-2 cells and to the mucus secreted by the HT29-MTX mucus-secreting cells. The bacteria interacted with the well-defined apical microvilli of Caco-2 cells without cell damage. The adhesion to Caco-2 cells of bifidobacteria did not require calcium and was mediated by a proteinaceous adhesion-promoting factor which was present both in the bacterial whole cells and in the spent supernatant of bifidobacterium culture. This adhesion-promoting factor appeared species specific, as are the adhesion-promoting factors of lactobacilli. We investigated the inhibitory effect of adhering human bifidobacterial strains against intestinal cell monolayer colonization by a variety of diarrheagenic bacteria. *B. breve* 4, *B. infantis* 1, and fresh human isolates were shown to inhibit cell association of enterotoxigenic, enteropathogenic, diffusely adhering *Escherichia coli* and *Salmonella typhimurium* strains to enterocytic Caco-2 cells in a concentration-dependent manner. Moreover, *B. breve* 4 and *B. infantis* 1 strains inhibited, dose dependently, Caco-2 cell invasion by enteropathogenic *E. coli*, *Yersinia pseudotuberculosis*, and *S. typhimurium* strains.

Lactobacilli and bifidobacteria are two species of the human normal microflora (13, 44) introduced into several fermented dairy products. These bacterial species are sometimes claimed to exert probiotic effects in humans, such as improving the properties of the indigenous flora (20, 25). As recently underlined by Klaenhammer (31) and Reid et al. (42), well-defined desirable properties should be carefully selected and characterized for specific use in commercial preparations. One of these properties is undoubtedly the adhesion to the mucosal surfaces which is known to be an important prerequisite for bacterial maintenance in the gastrointestinal tract. For example, recent reports concerning lactobacilli, which are nonpredominant organisms in the human gastrointestinal flora (13, 44), demonstrate that all strains do not possess the ability to adhere to human intestinal cells (10, 32). Selected strains such as *Lactobacillus acidophilus* BG2FO4 (8) and LB (7) and *Lactobacillus casei* GG (14) exhibit adhesive properties which allow interaction with the brush border of human polarized intestinal epithelial cell lines in culture. As observed by Elo and Salminen (14), lack of adhesion abilities of several bifidobacterial strains suggests that, as for lactobacilli, adhesive factors are not expressed by all *Bifidobacterium* strains.

To date little is known about adhesion of bifidobacteria to human intestinal cells. To gain further information, we used here the enterocyte-like Caco-2 (18, 41) and the mucus-secreting HT29-MTX (35) cell lines to investigate the adherence of a large number of human bifidobacterial strains. These well-characterized cellular models have been seen to display typical features of fluid-transporting and mucus-secreting intestinal cells (46). Bacterial attachment to the intestinal cell surfaces was characterized by light and electron microscopic examination and by quantitative determination. The mechanism through which the adhering strains interacted with eukaryotic cells was also investigated. Finally, since association with and invasion of the polarized Caco-2 cell line have been reported to mimic the in vivo conditions of infection by enterotoxigenic (11, 29, 30, 40) and enteroinvasive (12, 17, 19, 21, 33, 34, 37) bacteria and by viruses (45), we investigated here the competitive exclusion of diarrheagenic bacteria from Caco-2 cells by adhering bifidobacterial cells.

MATERIALS AND METHODS

Bacterial strains, growth conditions, and radiolabeling. Human bifidobacterial strains *Bifidobacterium breve* 4, 5, and 25; *Bifidobacterium longum* 4, 16, 18, and 22; *Bifidobacterium bifidum* 7 and 8; and *Bifidobacterium infantis* 1 (Nestec collection, Lausanne, Switzerland) and fresh human isolates from adults were grown in anaerobic conditions (GasPak H_2 + CO_2) in De Man-Rogosa-Sharpe (MRS) broth (Biokar, Pantin, France) twice for 24 h each time at 37°C before the adhesion assay. The enterovirulent bacterial strains (16, 36) used in this study were enterotoxigenic *Escherichia coli* (ETEC) H10407 expressing the CFA/I adhesive factor (15), enteropathogenic *E. coli* (EPEC) JPN15(pMAR7) (EAF^+ eae^+) (19) (J. B. Kaper, Center for Vaccine Development, University of Maryland), *Yersinia*

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pseudotuberculosis YPIII(pYV$^-$) (*inv*$^+$) (26) (M. Simonet, Faculté Necker-Enfants Malades, Paris, France), and *Salmonella typhimurium* SL 1344 (17) (B. A. D. Stocker, Stanford, California). Moreover, we used a diarrheagenic diffusely adhering *E. coli* (DAEC) C-1845 (1, 22), which belongs to the Dr family (38).

Before adherence assays, ETEC and DAEC strains were grown on CFA agar containing 1% Casamino Acids (Difco Laboratories, Detroit, Mich.), 0.15% yeast extract, 0.005% magnesium sulfate, and 0.0005% manganese chloride in 2% agar for 18 h at 37°C. EPEC bacteria were cultured at 37°C for 24 h in Luria broth. *S. typhimurium* was cultured at 37°C for 18 h in Luria broth. *Y. pseudotuberculosis* was cultured for 18 h at 28°C in Luria broth.

For radiolabeling, ETEC, DAEC, and EPEC bacteria were subcultured at 37°C twice for 24 h each time in Luria broth. They were metabolically labeled by the addition of [^{14}C]acetic acid (Amersham; 94 mCi/mmol, 100 μCi/10-ml tube) (ETEC and DAEC, 2.8×10^5 dpm/10^8 CFU/ml; EPEC, 1.9×10^5 dpm/10^8 CFU/ml). Before radiolabeling, *S. typhimurium* was cultured 4 h in Luria broth. For radiolabeling, they were subcultured at 37°C for 45 min in methionine medium (Difco) with [^{35}S]methionine (Amersham; 1,000 Ci/mmol, 20 μCi/ml) (*S. typhimurium*, 5.9×10^5 dpm/10^8 CFU/ml). After radiolabeling, bacteria were washed to remove the excess [^{14}C]acetic acid or [^{35}S]methionine.

Cell culture. The enterocyte-like Caco-2 cells were obtained from Jorgen Fogh (Sloan Kettering Memorial Cancer Center, Rye, N.Y.) (18, 41). The homogeneous mucus-secreting subpopulation of HT29-MTX cells (35) was obtained from A. Zweibaum (Unité 178, Institut National de la Santé et de la Recherche Médicale, Villejuif, France).

Cells were routinely grown in Dulbecco modified Eagle's minimal essential medium (25 mM glucose) (Eurobio, Paris, France) supplemented with 10% (HT29-MTX) or 20% (Caco-2) inactivated (30 min, 56°C) fetal calf serum (Boehringer, Mannheim, Germany) and 1% nonessential amino acids (Caco-2). For the adhesion assay, monolayers of Caco-2 cells and HT29-MTX cells were prepared on glass coverslips which were placed in six-well tissue culture plates (Corning Glass Works, Corning, N.Y.). For inhibition of intestinal cell colonization by pathogens, monolayers of Caco-2 cells were prepared in 24-well tissue culture plates (Corning). Cells were seeded at a concentration of 2×10^4 (HT29-MTX) and 1.4×10^4 (Caco-2) cells per cm^2. Maintenance of the cells and experiments were carried out at 37°C in a 10% CO_2–90% air atmosphere. The culture medium was changed daily. Mucus-secreting HT29-MTX and Caco-2 cells were used at between 20 and 40 and 60 and 90 cell passages, respectively. Cells were used for adherence assays at late postconfluence, i.e., after 15 days (Caco-2) and 20 days (HT29-MTX) in culture.

Adherence assay of bifidobacteria. The adherence of bifidobacterial strains to Caco-2 and HT29-MTX cells was examined as described previously for *L. acidophilus* LB and *L. acidophilus* BG2FO4 adhesion assays (7, 8). Briefly, the Caco-2 and HT29-MTX monolayers, prepared on glass coverslips which were placed in six-well Corning tissue culture plates, were washed twice with phosphate-buffered saline (PBS). Bifidobacteria (1 ml, 4×10^8 bacteria per ml in spent culture supernatant, treated supernatant, or fresh MRS broth) were added to 1 ml of the cell line culture medium. This suspension (2 ml) was added to each well of the tissue culture plate, and the plate was incubated at 37°C in 10% CO_2–90% air. After 1 h of incubation, the monolayers were washed five times with sterile PBS, fixed with methanol, stained with Gram stain, and examined microscopically. Each adherence assay was conducted in duplicate over three successive passages of intestinal cells. For each monolayer on a glass coverslip, the number of adherent bacteria was evaluated in 20 random microscopic areas. Adhesion was evaluated by two different technicians to eliminate bias.

In some adherence assays *L. acidophilus* LB (7) and *L. acidophilus* BG2FO4 (8) were used as controls of positive adhesion.

Physical and chemical treatments of bacteria and spent culture supernatants. All enzymes and chemicals were obtained from Sigma Chemical Co. (St. Louis, Mo.). Bacterial cells and spent culture supernatant were separated by centrifugation (20,000 × *g*, 1 h at 4°C). Bacterial cells alone or spent broth culture supernatant alone was incubated with trypsin or pronase (2.5 mg ml^{-1}) for 60 min at 37°C and then inactivated by adding inactivated (30 min, 56°C) fetal bovine serum (Boehringer). To determine the influence of calcium (7, 8, 32) on adherence of bifidobacteria, the monolayers were washed five times with the chelating agent of calcium [ethylene-bis(oxyethylenenitrilo)tetraacetic acid (EGTA)] at 20 mM in PBS, after the incubation period with bacteria.

In experiments performed to study species specificity of extracellular adhesins, the spent culture supernatants of BG2FO4 and LB lactobacilli were obtained by centrifugation (20,000 × *g*, 1 h at 4°C) and added to the bifidobacterial cells.

Scanning electron microscopy. For scanning electron microscopy, the tissue culture cells were grown on glass coverslips. After the bacterial adhesion assay, cells were fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for 1 h at room temperature. After two washes with phosphate buffer, cells were postfixed for 30 min with 2% OsO_4 in the same buffer, washed three times with phosphate buffer, and dehydrated in a graded series (30, 50, 70, 80, 90, and 100%) of ethanol. Cells were dried in a critical-point dryer (Balzers CPD030) and coated with gold. The specimens were then examined with a Jeol JSM 25S scanning electron microscope.

Pathogen cell association and cell invasion inhibition assay. Before adhesion and invasion inhibition assays, the Caco-2 cell monolayers were washed twice with PBS.

To determine Caco-2 cell-associated pathogens (adhering plus invading bacteria), radiolabeled bacteria were used (6). Radiolabeled pathogens were suspended in their culture medium (see Bacterial strains, growth conditions, and radiolabeling section above), and 1 ml (10^8 CFU/ml) of this suspension was added to each Caco-2 cell culture well. For ETEC, DAEC, and EPEC, incubations were conducted in the presence of 1% D-mannose that inhibits type 1-mediated pilus adhesion. The plates were incubated at 37°C in 10% CO_2–90% air for 60 min for ETEC, DAEC, and *Salmonella* spp. and for 180 min for EPEC. The monolayers were then washed three times with sterile PBS. Cell-associated bacteria and intestinal cells were dissolved in a 0.2 N NaOH solution. The level of bacterial adhesion was evaluated by liquid scintillation counting.

Cell entry of pathogens was determined by quantitative determination of bacteria located within the Caco-2 cells by using unlabeled bacteria and an aminoglycoside antibiotic (21, 26). Bacteria were suspended in their culture media, and 2 ml (10^8 CFU/ml) of this suspension was added to each Caco-2 culture well. The plates were incubated at 37°C in 10% CO_2–90% air for 180 min for EPEC, for 90 min for *Yersinia* spp., and for 60 min for *Salmonella* spp. For *Yersinia* spp., contact with Caco-2 cells was prolongated by centrifugation (10 min at 123 × *g*). After incubation, the

TABLE 1. Adhesion of human bifidobacterial strains to human intestinal epithelial Caco-2 cells in culture

Bifidobacterial strain	Adhesion[a]	
	Without EGTA	With 20 mM EGTA
B. breve		
4	210 ± 20	205 ± 18*
5	75 ± 12	37 ± 8**
25	41 ± 5	8 ± 4**
B. longum		
4	115 ± 12	70 ± 10**
16	153 ± 23	72 ± 13**
18	75 ± 10	30 ± 8**
22	28 ± 5	5 ± 3**
B. bifidum		
7	81 ± 7	30 ± 2**
8	78 ± 10	35 ± 6**
B. infantis 1	161 ± 14	137 ± 17*
Fresh human isolates		
20	155 ± 21	135 ± 14*
28	160 ± 14	133 ± 18*
29	163 ± 17	135 ± 18*

[a] Adhesion of bifidobacterial strains (2×10^8 CFU/ml) onto monolayers of differentiated Caco-2 cells on 20 randomized microscopic fields per coverslip was evaluated. The figures represent mean numbers ± standard deviations of bifidobacteria adhering per 100 Caco-2 cells. Each experiment was conducted in duplicate. The variability between the duplicates was less than 5%. Each adherence assay was conducted with three successive Caco-2 cell passages. Statistical analysis between adhesion with EGTA versus adhesion without EGTA was performed by the Student *t* test. *, no significant difference; **, $P < 0.01$.

plates were washed three times with sterile PBS and afterwards incubated for 120 min in a medium containing 20 to 100 μg of gentamicin per ml. Since gentamicin does not diffuse into the cells, bacteria that adhere to the Caco-2 brush border were rapidly killed, whereas those located within Caco-2 cells were not killed. The monolayer was washed with PBS and lysed with sterilized H_2O. Appropriate dilutions were plated to determine the number of viable intracellular bacteria.

For evaluation of inhibition of cell association of pathogens by bifidobacteria, 1 ml of radiolabeled pathogen (10^8 CFU/ml) and 1 ml of bifidobacteria with spent culture supernatant (10^9 to 10^7 CFU/ml, as indicated) were added together to each culture well and incubated as previously described (6). For evaluation of inhibition of cell entry of pathogens by bifidobacteria, 1 ml of unlabeled pathogen (10^8 CFU/ml) and 1 ml of bifidobacteria with spent culture supernatant (10^8 or 10^7 CFU/ml, as indicated) were added together to each culture well and incubated as previously described (6). Each assay was conducted in triplicate with three successive passages of Caco-2 cells.

RESULTS

Adhesion of human bifidobacteria to enterocyte-like Caco-2 and to mucus-secreting HT29-MTX cell lines. Thirteen human bifidobacterial strains were examined for their abilities to adhere to polarized human intestinal epithelial Caco-2 cells (Table 1). Bifidobacterial strains expressed various adhesiveness capacities randomly distributed among the variety of *Bifidobacterium* species tested. Low attachment to the differentiated Caco-2 cells was observed with *B. breve* 5 and 25, *B. longum* 18 and 22, and *B. bifidum* 7 and 8. Moderate attachment was observed with *B. longum* 4. High attachment was observed with *B. breve* 4, *B. longum* 16, and *B. infantis* 1. It was noticed that all three fresh human isolates from adults possessed a high calcium-independent capacity of adhesion. As for lactobacilli (7, 8, 32), we studied here the attachment of bifidobacterial strains to discriminate between calcium-dependent and calcium-independent adhesion. Washing with EGTA after the adhesion assay decreased significantly the attachment of *B. longum* 16 and *B. infantis* 1. By contrast, the rinses with EGTA had no effect on the adherence of *B. breve* 4. This result showed that among the adhering bifidobacterial strains tested here, only the *B. breve* 4 has a high calcium-independent capacity to bind to differentiated Caco-2 cells in culture. It is interesting to note that attachment of *B. breve* 4 appears slightly greater than that of *L. acidophilus* LB (7) and BG2FO4 (8). Examination of *B. breve* 4 and *B. infantis* 1 whole cell adhesion was also performed by scanning electron microscopy (Fig. 1). Scanning electron micrographs clearly illustrated that binding of the bacteria to Caco-2 cells occurred at the mucosal surface (Fig. 1A). Bifidobacteria interact with the brush border of the differentiated Caco-2 cells (Fig. 1B). At a concentration of 10^9 CFU/ml, the high level of adhesion obscured the brush border, which is present under the biofilm of bifidobacteria (Fig. 1C). The biofilm of bifidobacteria was constituted by bacteria adherent to each other (Fig. 1D).

The strain *B. breve* 4 also adhered to the mucus-secreting HT29-MTX cell line, which is a homogeneous subpopulation of goblet cells (Fig. 2). *B. breve* 4 whole cells conspicuously both interacted with the secreted mucus (Fig. 2A) and adhered to each other (Fig. 2B). It was observed that the level of adhesion of *B. breve* 4 whole cells to the mucus-secreting intestinal cells (Fig. 2A) appeared higher than adhesion to enterocyte-like Caco-2 cells (Fig. 1A).

Characterization of bifidobacterial adhesion. In an attempt to identify the bifidobacterial components involved in microbial adhesion to enterocyte-like cells, we carried out experiments using the highly adhering strain *B. breve* 4. We subjected the bacteria and their spent culture medium to substitution and enzymatic treatments (Table 2), as previously done for adhesion of lactobacilli (7, 8). When the spent culture supernatant was discarded and replaced by a fresh culture medium, a reduction in bacterial adhesion occurred. Moreover, trypsin or pronase treatments of the bacteria with the spent culture supernatant almost totally abolished the adhesiveness of *B. breve* 4. This result indicated that a proteinaceous component is involved in the adhesion of *B. breve* 4.

We have observed with lactobacilli that spent culture supernatant of *L. acidophilus* BG2FO4 could promote adhesion of the moderately adhering *L. casei* GG (8). To study the species specificity of the extracellular adhesion promoting factor of *B. breve* 4, we conducted additional experiments with spent culture supernatants from *L. acidophilus* LB and BG2FO4. Results in Table 3 show that the spent culture supernatants of *L. acidophilus* LB and BG2FO4 did not favor adhesion of *B. breve* 4. Conversely, the spent culture supernatant of *B. breve* 4 did not increase adhesion of *L. casei* GG. This result indicates a strong species specificity of the subcellular adhesion-promoting factors of lactobacilli and bifidobacteria.

Inhibition by human bifidobacterial strains of Caco-2 cell colonization by enterovirulent bacteria. Monolayers of Caco-2 cells were infected apically with ETEC H10407

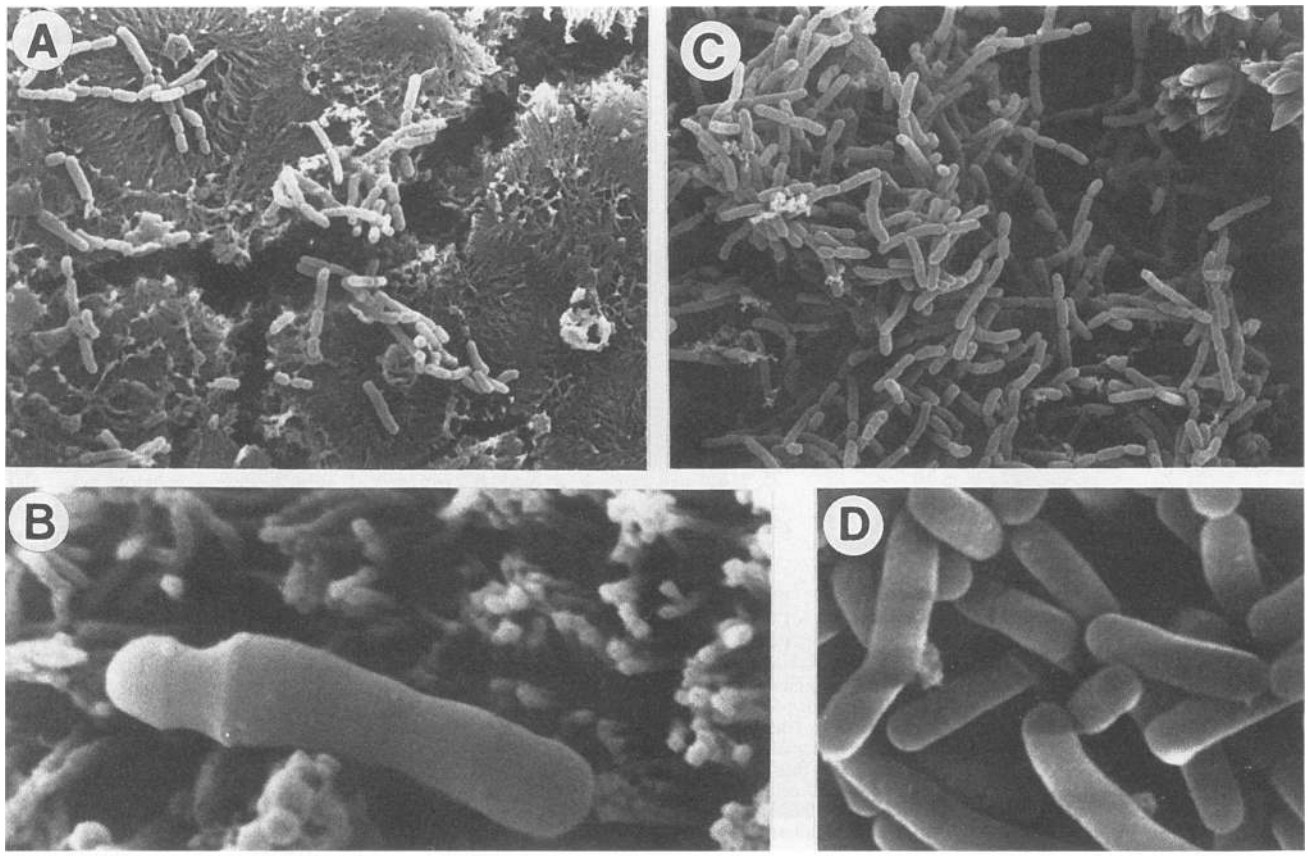

FIG. 1. Observation by scanning electron microscopy of the adherence of human *B. breve* 4 and *B. infantis* 1 onto the brush border of the polarized human intestinal epithelial Caco-2 cells. (A) Low magnification showing adhesion of *B. breve* 4 (10^8 CFU/ml) onto the brush border of Caco-2 cells (magnification, ×2,000); (B) High magnification showing interaction of *B. infantis* 1 (10^8 CFU/ml) with brush border of Caco-2 cells (magnification, ×20,000); (C and D) Low and high magnifications, respectively, showing *B. breve* 4 (10^9 CFU/ml) which covers the surface of Caco-2 cells (respective magnifications, ×2,000 and ×10,000).

bearing the CFA/I adhesive factor, DAEC C-1845, EPEC JPN15(pMAR7), *S. typhimurium* SL 1344, and *Y. pseudotuberculosis* YPIII (pYV$^-$). Using radiolabeled bacteria (10^8 CFU/ml), we have quantified the cell associations (adhering plus invading bacteria) with differentiated Caco-2 cells (Table 4). Using unlabeled bacteria (10^8 CFU/ml) and the gentamicin assay, we quantified the bacterial entry into differentiated Caco-2 cells (Table 5). Inhibition of both cell association (Table 4 and Fig. 3) and cell invasion (Table 5) by adhering *B. breve* 4, *B. infantis* 1, *B. longum* 4 and 16, and fresh human clinical isolates of *Bifidobacterium* strains 20, 29, and 28 were examined. Adhering strains inhibited Caco-2 cell association and cell invasion of all pathogens tested. As observed in Fig. 3, the highly adhering strain *B. breve* 4 inhibited dose dependently the cell association of ETEC, DAEC, EPEC, and *S. typhimurium*. For all pathogens (10^8 CFU/ml), 50% inhibition was obtained with 10^8 CFU of bifidobacteria per ml.

Invasion of Caco-2 cells by *Y. pseudotuberculosis*, *S. typhimurium*, and EPEC (10^8 CFU/ml) was dose dependently inhibited by adhering *B. breve* 4 and *B. infantis* 1 (Table 5). *B. breve* 4 more efficiently inhibited Caco-2 cell invasion by EPEC than did *Y. pseudotuberculosis* and *S. typhimurium*.

DISCUSSION

Probiotics are defined as live microorganisms which when administered to humans or animals, as dried cells or fermented products, affect beneficially the host by improving the properties of the indigenous microflora (39). This definition, although not restricted to bifidobacteria and lactobacilli, highlights the importance of these latter genera, which are the most commonly used in the dairy industry. With this regard, questions related to the selection of the most promising probiotic strains must be posed (31). Recently, Klaenhammer (31) and O'Sullivan et al. (39) have proposed to select probiotics on the basis of sound technological and biological criteria. One of these is clearly adhesion to intestinal mucosa.

Two major cell phenotypes, i.e., enterocytes and goblet cells, are represented in the intestinal mucosa. We have used two well-characterized cultured colon carcinoma cell lines to study adhesion of human bifidobacteria. The human Caco-2 cell line displays typical features of enterocytic differentiation and has been extensively used to study the organization and function of human intestinal cells (46). Moreover, since the human small intestinal mucosa has a mucus coating at the mucosal surface, the homogeneous mucus-secreting cell

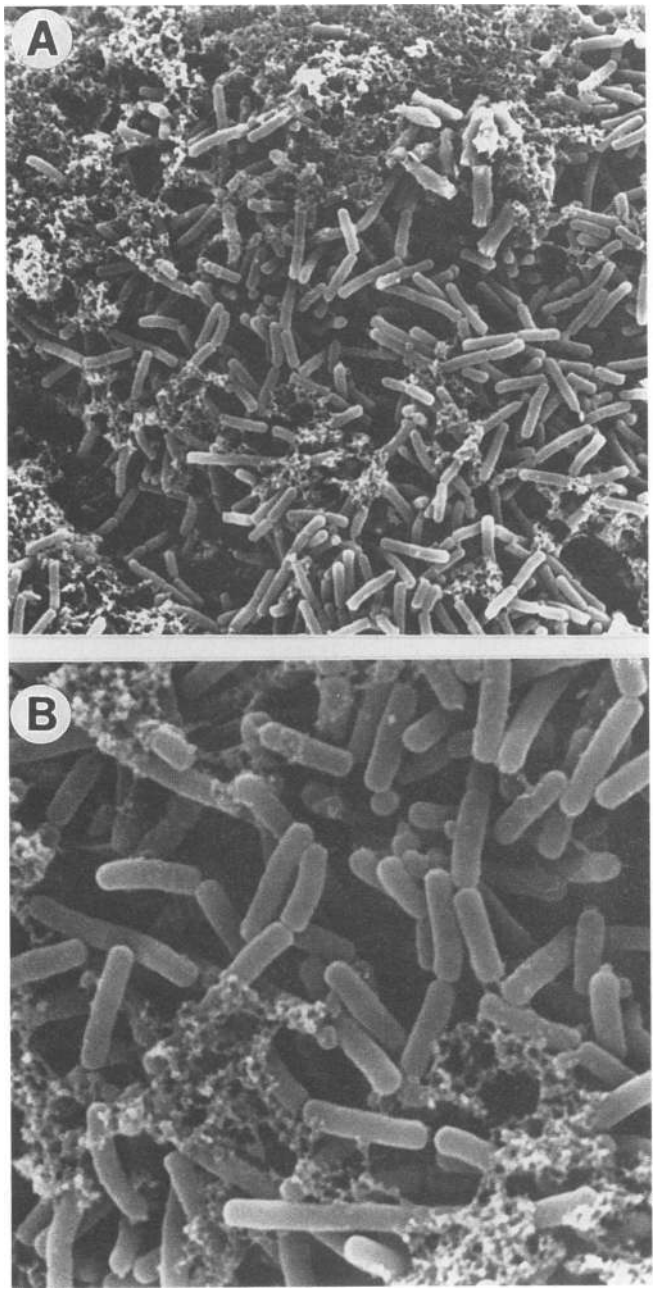

FIG. 2. Adhesion of human *B. breve* 4 to human mucus-secreting HT29-MTX cells observed by scanning electron microscopy. Notice that *B. breve* 4 whole cells (10^8 CFU/ml) interact with the mucus secreted by the HT29-MTX cells (magnification, ×2,000 [A] and ×5,000 [B]).

population HT29-MTX (35) is an appropriate human goblet cell model.

It should be noted that Caco-2 cells have already been shown to behave as an ideal model to mimic host-pathogen interactions in the gut. Thus, by demonstrating here that some but not all human bifidobacteria express adhesive properties to Caco-2 cells, our results, along with those of Elo and Salminen (14), propose a new tool for selecting bifidobacteria for health food products. Even if the in vivo relevance of such a selection criterion remains to be established, it has been recently observed that *Bifidobacterium* spp. consumed in a fermented dairy product could be recovered in a high number from the stools of human volunteers (3). Upon cessation of intake, the bacterial levels gradually decreased to zero during an 8-day period, indicating that the strain persisted in the gut for a period longer than the normal transit, perhaps because of intimate relationships between bacteria and the gut mucosa.

An important function of the microflora is to exert a barrier against colonization of the gastrointestinal tract by pathogenic bacteria (9, 13, 42). Adhesion of lactobacilli (7, 8, 14, 32) and competitive exclusion of pathogens from intestinal (6) and uroepithelial (4, 5, 43) cells and mucus (2) have been recently well documented. Moreover, the administration of high doses of a selected strain of *L. casei*, which adheres to Caco-2 cells (14), favors the maintenance of this bacterium in the human intestinal tract (23). This was even clinically effective in reducing the time of recovery in cases of diarrhea in children (27). Previous results strongly suggest

TABLE 2. Characterization of adhesion of the human strain *B. breve* 4

Condition	Adhesion[a]
With spent culture supernatant	210 ± 18
With a fresh culture medium[b]	105 ± 12*
With PBS	97 ± 18*
After trypsin pretreatment[c]	30 ± 5*
After pronase pretreatment[c]	27 ± 10*

[a] Adhesion scores in 20 randomized microscopic fields per coverslip were determined. The figures represent mean numbers ± standard deviations of bifidobacteria adhering to the cell monolayer per 100 Caco-2 cells. Each adherence assay was conducted in duplicate with three successive Caco-2 cell passages. Statistical analysis was performed by the Student *t* test. *, $P < 0.01$ versus adhesion of *B. breve* 4 with its spent culture supernatant.

[b] To determine the presence of adhesive factors secreted by the bacteria into the spent culture supernatant, it was replaced by a fresh culture medium or PBS before the adhesion assay.

[c] To further characterize the bacterial determinants involved in *B. breve* 4 adhesion, bacteria with spent broth culture supernatant were subjected to trypsin or pronase treatments (2.5 mg/ml) for 60 min at 37°C and then inactivated by adding inactivated (30 min, 56°C) fetal bovine serum. As a control, inactivated fetal bovine serum added to *B. breve* 4 with its spent culture supernatant did not modify adhesion of *B. breve* 4 (205 ± 28 bacteria per 100 Caco-2 cells).

TABLE 3. Involvement of species-specific secreted components in bifidobacterial adhesion to Caco-2 cells in culture

Bacterial strain and condition	Adhesion[a]
B. breve 4	
With spent culture supernatant	215 ± 22
With spent culture supernatant of *L. acidophilus* LB	70 ± 12*
With spent culture supernatant of *L. acidophilus* BG2FO4	80 ± 7*
L. casei GG	
With spent culture supernatant	60 ± 15*
With spent culture supernatant of *B. breve* 4	57 ± 14*

[a] Bacterial cells and spent culture supernatant were separated by centrifugation (20,000 × *g*, 30 min). Adhesion scores in twenty randomized microscopic fields per coverslip were determined. The figures represent mean numbers ± standard deviations of bifidobacteria adhering to the cell monolayer per 100 Caco-2 cells. Each adherence assay was conducted in duplicate with three successive Caco-2 cell passages. Statistical analysis was performed with a Student test. *, $P < 0.01$ versus adhesion of *B. breve* 4 with its spent culture supernatant.

TABLE 4. Cell association of enterovirulent bacteria with Caco-2 cells and inhibition of cell association by adhering human bifidobacterial strains

Bifidobacterium (CFU/ml)	% Inhibition of cell association[a]			
	ETEC	DAEC	EPEC	*S. typhimurium*
B. infantis 1				
10^9	58 ± 6	53 ± 8	77 ± 2	82 ± 3
10^8	18 ± 2	28 ± 4	30 ± 6	23 ± 9
B. longum 4				
10^9	59 ± 6	71 ± 4	83 ± 2	88 ± 3
10^8	29 ± 2	30 ± 4	39 ± 4	7 ± 3
B. longum 16				
10^9	47 ± 7	56 ± 5	72 ± 3	62 ± 12
10^8	14 ± 2	41 ± 10	33 ± 5	0
Fresh human isolates				
Strain 20				
10^9	58 ± 7	54 ± 3	77 ± 4	81 ± 2
10^8	7 ± 2	30 ± 4	38 ± 5	4 ± 3
Strain 28				
10^9	55 ± 3	55 ± 4	76 ± 4	75 ± 14
10^8	38 ± 3	17 ± 2	43 ± 3	39 ± 6
Strain 29				
10^9	48 ± 4	51 ± 2	64 ± 1	65 ± 5
10^8	35 ± 2	27 ± 7	43 ± 6	2 ± 0.8

[a] Associated bacteria (adhering plus invading) with differentiated Caco-2 cells were measured after incubating radiolabeled bacteria at 37°C in 10% CO_2–90% air for 60 min for ETEC, DAEC, and *S. typhimurium* and 180 min for EPEC, all at 10^8 CFU/ml. Each experiment was conducted in triplicate. The data represent mean values of experiments from three successive passages of Caco-2 cells. Cell association for the enterovirulent bacteria was as follows: ETEC, 2.5% ± 0.3%; DAEC, 1.4% ± 0.2%; EPEC, 11.0% ± 0.5; and *S. typhimurium*, 3.1% ± 0.2%. Cell association values are percentages of incubated bacteria.

that facultative anaerobes could exert antagonistic effects in the small intestine, whereas strict anaerobes of the physiological microbial flora exert activities in the large intestine and in the colon. Indeed, a recent in vivo study by Itoh and Freter in gnotobiotic mice showed that lactobacilli can compete with *E. coli* in the stomach and the small intestine, whereas *Clostridium* spp. have been found to control *E. coli* in the large intestine (28). Since bifidobacteria are members

TABLE 5. Adhering human bifidobacterial strains inhibit cell invasion of enterovirulent bacteria within Caco-2 cells

Bifidobacterium (CFU/ml)	% Inhibition of cell invasion[a]		
	EPEC	*Y. pseudotuberculosis*	*S. typhimurium*
B. breve 4			
10^8	93 ± 2	64 ± 19	42 ± 6
10^7	36 ± 6	17 ± 3	10 ± 2
B. infantis 1			
10^8	ND	ND	33 ± 1
10^7	ND	ND	13 ± 6

[a] Invaded bacteria within undifferentiated (*Y. pseudotuberculosis*) and differentiated (EPEC and *S. typhimurium*) Caco-2 cells were measured after incubating unlabeled bacteria at 37°C in 10% CO_2–90% air for 60 min for *Y. pseudotuberculosis* and *S. typhimurium* and 180 min for EPEC, all at 10^8 CFU/ml. The monolayers were then washed three times with sterile PBS and incubated for 120 min in medium containing 20 to 100 μg of gentamicin per ml to kill extracellular bacteria. Each experiment was conducted in triplicate. The data represent mean values ± standard deviations of experiments from three successive passages of Caco-2 cells. ND, not determined. Cell invasion (as a percentage of incubated bacteria) was as follows: EPEC, 1.5% ± 0.4%; *Y. pseudotuberculosis*, 8.5% ± 0.9%; and *S. typhimurium*, 8.2% ± 1.5%.

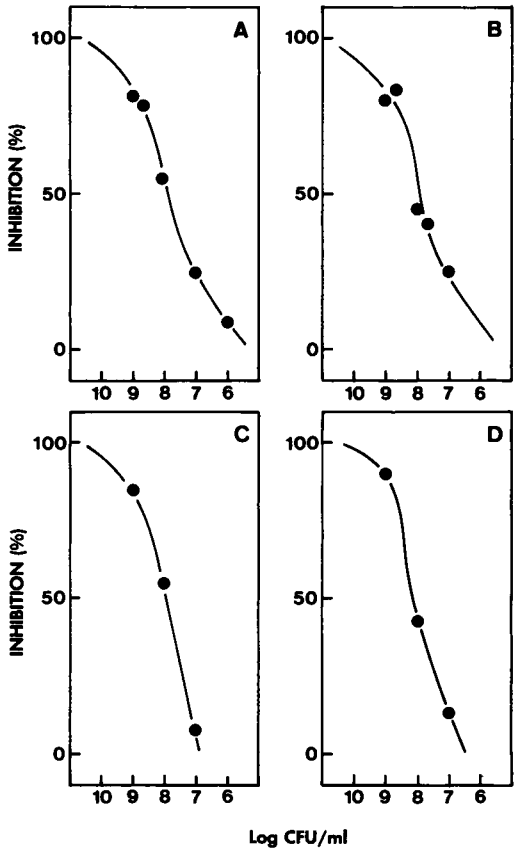

FIG. 3. Competitive exclusion of diarrheagenic bacteria from human enterocyte-like Caco-2 cells in culture by human *B. breve* 4. (A) ETEC; (B) EPEC; (C) DAEC; (D) *S. typhimurium* (10^8 CFU/ml in each panel). Each experiment was conducted in triplicate. The data represent mean values of experiments from three successive passages of Caco-2 cells (standard deviations, not shown, were less than 5%).

of the dominant microflora, it may be more relevant to use this genus as a probiotic, especially in newborns and young infants.

We analyzed the mechanism of adhesion of *B. breve* 4, as already described for lactobacilli (7, 8). We report here the occurrence of bifidobacteria adhering to the human intestinal cells by a mechanism of adhesion which involves a proteinaceous component. Moreover, we observed that the adhesion was decreased by 50% when the spent culture supernatant of *B. breve* 4 was discarded. These results show that the adhesive factor of *B. breve* 4 is a proteinaceous component present both in the spent culture supernatant and on the bacterial cell surface. These results suggest that bifidobacteria could adhere by a labile surface-associated proteinaceous component. This mechanism of adhesion appears different from that of lactobacilli, which adhere by a secreted proteinaceous component (7, 8, 24). The adhesin-like protein(s) of bifidobacteria seems to be species specific, since adhesion of bifidobacteria is not promoted by spent supernatants of adhering lactobacilli. Nevertheless, it should be feasible to take advantage of the good adhesive properties of these human bifidobacteria which could confer to these organisms a competitive advantage in vivo. Interestingly, we also show that adhesive strains of bifidobacteria can inhibit adhesion of enteropathogens significantly, although the

mechanism remains unclear. Our results do not explain whether the pathogens' exclusion is due to a competition for specific sites on the enterocytic cell surface or to the constitution of a biofilm of bacteria preventing the access to the cell surface of enterovirulent organisms. These results show a potential for adhesive human bifidobacteria in inhibiting the cell association and the cell entry of human enteropathogens. Bifidobacteria have been introduced into several fermented dairy products. Our results suggest that adhering human bifidobacteria, as recently demonstrated for adhering *L. casei* GG (14, 23, 27), could prevent diarrhea. This hypothesis, of course, still needs to be proved in vivo by human clinical studies.

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