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ORIGINAL ARTICLE

The role of hemagglutination and effect of exopolysaccharide production on bifidobacteria adhesion to Caco-2 cells *in vitro*

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ABSTRACT

It is believed that an important criterion for a potential probiotic strain is that it is capable of adhering to mucosal surfaces in the human gastrointestinal tract. The purpose of this study was to investigate a possible relationship between exopolysaccharide production and adhesion to Caco-2 cells by *Bifidobacterium breve* A28 and *Bifidobacterium bifidum* A10. In a preselection process, the hemagglutination abilities of these bacteria were determined prior to undertaking adhesion studies. *B. breve* A28, which produces large amounts of EPS (97.00 ± 2.00 mg/l) and has good hemagglutination abilities (+3) was found to adhere strongly to Caco-2 cells. Under gastrointestinal conditions, the high EPS producing- *B. breve* A28 was found to have better viability and adhesion to Caco-2 cells than the low EPS producing- *B. bifidum* A10. Also, *B. breve* A28 was found to be more effective at inhibiting *Escherichia coli* ATCC 11229 than *B. bifidum* A10. This investigation showed that high EPS production and adhesion ability may be important in the selection of bifidobacteria as probiotic strains.

Key words adhesion, bifidobacteria, exopolysaccharide production, hemagglutination.

Some species and strains of the genus *Bifidobacterium* are considered to be probiotic and are used as active ingredients in functional food products. These microorganisms have numerous health-promoting attributes (1). To exert beneficial effects, they must overcome biological barriers, including acid in the stomach and bile in the intestine, in order to at least temporarily colonize specific parts of the intestinal tract (2). As well as the above properties, EPS production by these bacteria is important to their viability. A protective coating of EPS may allow the bacteria to better withstand stomach acid and bile salts. In addition, EPS production may improve adherence to the intestinal mucosa and increase longevity in the intestinal tract (3, 4). However, as far as we know, there are no studies of the factors which influence adhesion of exopolysaccharide producing-*Bifidobacterium* strains to intestinal cells. The effectiveness of these organisms is related to their ability to interact with and adhere to the intestinal wall. The ability to adhere is a prerequisite for bacterial colonization; it is also essential for the balance of intestinal microflora, intestinal bacterial enzyme activity and stabilization of intestinal permeability (5). Therefore, mucosal adhesion has been proposed as one of the main selection criteria for probiotic strains (6). In addition, it may contribute to the ability to competitively exclude enteropathogens.

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List of Abbreviations: ANOVA, one-way analysis of variance; ATCC, American Type Culture Collection; *B. bifidum Bifidobacterium bifidum*; *B. breve Bifidobacterium breve*; CFU, colony forming unit; DMEM, Dulbecco's modified Eagle's minimal essential medium; ETEC, enterotoxigenic *Escherichia coli*; *E. coli Escherichia coli*; EPS, exopolysaccharide; HA, hemagglutination; MRS, Man Rogosa-Sharpe; MRSC, Man Rogosa-Sharpe with L-cysteine hydrochloride; OD, optical density; RBC, red blood cells.

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Several probiotics have been shown to inhibit pathogen adhesion (7–9). This could be the mechanism of probiotic protection against ETEC infection, which otherwise attaches to mucosal surfaces in order to release the toxins responsible for the development of diarrhea and inflammation (10). However, there is increasing evidence that probiotics may act, not only by competing for pathogen adhesion, but also through other diverse mechanisms, including maintenance of mucosal barrier integrity and modulation of mucosal immune response (11–13). However, *in vivo* studies of bacterial adhesion are not easy to perform while *in vitro* model systems, mainly based on the use of human enterocyte-like Caco-2 cells, are expensive and time-consuming (14). Thus, it is imperative to select such strains and further improve their adhesion ability by optimizing their growth conditions, thus enhancing colonization by them (15). Because of the complexity of *in vitro* and *in vivo* studies, some researchers have suggested that HA testing should be undertaken prior to adhesion assays. HA is recommended as an adhesion test on the basis that the structure of the receptors of epithelial mammalian cells is similar to that of erythrocytes. This ability has been correlated with the presence of lectins on the surface of the bacteria (16). Many enterotoxigenic bacteria have been shown to colonize the intestinal surface via fimbrial adhesions that frequently have lectin-like activities and bind to epithelial glycoconjugate receptors (17–19).

The aims of this study were to investigate two desirable properties for probiotic microorganisms (i) resistance to gastric conditions and (ii) ability to adhere to mucosal surfaces. The adhesion to Caco-2 cells of bifidobacteria which had been determined to have both hemagglutination ability and EPS production, and the effects of gastrointestinal conditions on the adhesion ability of these bacteria were examined. In addition, the inhibitory effects on *E. coli* ATCC 11229 of *B. breve* A28 and *B. bifidum* A10 were determined and related to their EPS production.

MATERIAL AND METHODS

Microorganisms and growth conditions

B. breve A28 and *B. bifidum* A10 strains were isolated from breast-fed human feces during a previous study, and obtained from the stock collection of the Biotechnology Laboratory at Gazi University, Faculty of Science and Arts, Department of Biology (Ankara, Turkey) (20). These strains were classified by their morphologic and cultural properties, catalase test (negative), and the API 20A kit analyzed by API LAB plus software version 4.0 databases (bioMerieux, Lyon, France). A28 and A10 were activated anaerobically at 37°C for 24–48 hr in MRS broth (Merck, Darmstadt, Germany) with L-cysteine hydrochloride (0.05%) before experimental use. MRSC agar plates plated with inoculates taken from different dilutions were incubated for 48 hr at 37°C in anaerobic conditions. Anaerobic conditions were created by Anaerocult A gas packs (Merck, Darmstadt, Germany). *E. coli* ATCC 11229 was obtained from the culture collection held at the Refik Saydam Hygiene Centre, Ankara, Turkey. *E. coli* ATCC 11229, which was used as a negative control, was maintained on nutrient agar (Oxoid, Cambridge, UK) and cultured in nutrient broth overnight at 37°C for 24 hr before use. Active *B. breve* A28, *B. bifidum* A10 and *E. coli* ATCC 11229 strains were grown statically in broth at 37°C and harvested by centrifugation (10 000 × *g*, 15 min) in the late exponential growth phase. Cultures were washed twice with 50 mM KH_2PO_2-Na_2HPO_4, containing 8 g/l NaCl and 2 g/l KCl, and pH 7.2 PBS, and finally resuspended in the same buffer. Bacterial concentration was adjusted to $OD_{600} = 0.600$.

EPS production and hemagglutination ability of *B. breve* A28 and *B. bifidum* A10

MRSC (with L-cysteine hydrochloride) broth cultures were used for cultivation of the strains and investigation of EPS production by these strains. After inoculation, the broth cultures were incubated at 37°C for 18 hr. After incubation, the cultures were boiled at 100°C for 10 min. After cooling, 17% (v/v) of 85% trichloroacetic acid solution was added and the resultant mixture centrifuged (21). Thus separation of cells and protein was achieved by centrifugation. EPS was precipitated with ethanol (100% v/v), and then recovered by centrifugation at 4°C at 14 000 rpm for 20 min. Total EPS (mg/l) was estimated in each sample by the phenol-sulfuric method (22) using glucose as standard (23). All experiments were done in triplicate and the mean values calculated.

HA using gel technology was determined by the following assay. The gel tests use the principle of controlled centrifugation of RBC through a dextran-acrylamide gel (Diamed AG 1785, Cressier-sur-Morat, Switzerland), appropriate reagents being pre-prepared in a specially designed micro tube (24). The cells were inoculated into MRSC broth and subcultured for 16–18 hr at 37°C twice before further studies. The cultures were adjusted at MacFarland 8. Bifidobacteria cultures (125 μl) were mixed with 25 μl of RBC from human type O secretor (0.08% suspensions) (DiaMed AG). Micro tubes were centrifuged at 1030 rpm for 10 min and the HA ability was measured visually. The results were recorded as +3 and 0 (negative HA).

Antimicrobial testing

The antimicrobial activity of the *B. breve* A28 and *B. bifidum* A10 used in this work was tested against *E. coli*

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ATCC 11229 using the assay described by Lievin *et al.* (25). Briefly, nutrient agar plates containing *E. coli* ATCC 11229 at a concentration of 10^7 CFU/ml were prepared. A 3 mm diameter gel punch was used to make four evenly spaced wells per plate. Samples were added to each well. Plates were incubated for 3 hr at 37°C under anaerobic conditions then overlaid with 10 ml sterile agar. After incubation for 18–24 hr at 37°C under anaerobic conditions, the diameter of the inhibition area surrounding the wells was measured.

Cell lines

The Caco-2 cell line was originally isolated from a human colon adenocarcinoma and obtained from the cell and virus bank of the Foot- and Mouth Disease Institute (Ankara) of the Ministry of Agriculture and Rural Affairs of Turkey. The cells were cultured in DMEM (Flow, Mississauga, ON, Canada) supplemented with 10% (v/v) heat inactivated fetal calf serum, 2 mM L-glutamine (Sigma, Farma, Turkey), 100 IU/ml penicillin and 100 μg/ml streptomycin (Flow). This was cultured in 25 cm^2 cell culture bottles in an incubator with 10% CO_2/90% air at 37°C. For the adhesion assays Caco-2 monolayers were placed in 24-well tissue culture plates. Cells were seeded at a concentration of 1×10^5 cells per well to obtain confluence and maintained for two weeks before use in adhesion assays. The cell culture medium was changed every other day and replaced by fresh DMEM. Caco-2 monolayers were washed twice with 1 ml of sterile PBS before the adhesion assay.

Adherence assays

Adherence assays were performed by adding 1 ml (1×10^7 CFU/ml) of the bacterial strains *B. breve* A28, *B. bifidum* A10 and *E. coli* ATCC 11229 in spent culture supernatant MRSC or nutrient to 1 ml of the cell-line growth medium. This mixture was added to a washed monolayer of cells on cover slips placed in a twenty four-well tissue culture dish, and incubated for 1 hr at 37°C in 10% CO_2/90% air. The monolayers were washed twice with PBS (pH 7.4), fixed in methanol, Gram stained and examined microscopically under an oil immersion lens. Adherence was evaluated in 50 random microscopic fields and the mean ± standard deviation of adhering bacteria per 100 cells of epithelial cell-line was determined.

The results of adhesion assays are expressed as "adhesion index" for each strain. The adhesion index is the numbering of adherent bacterial cells per 100 epithelial cells.

Competitive adhesion

In order to study competition for adhesion on Caco-2 cells, *B. breve* A28, *B. bifidum* A10 and *E. coli* ATCC 11229 strains were added simultaneously (1:1 v/v) to the Caco-2 cultures before counting. The free cells of these bacteria were removed by washing with PBS (pH 7.2) fixed with methanol, Gram stained and examined microscopically.

Scanning electron microscopy

Bacterial attachment was also determined qualitatively by electron microscopic examination. Cultured cells grown on glass cover slips were used for scanning electron microscopic studies. After the bacterial adhesion assay, the cells were fixed with 3% glutaraldehyde (Sigma) in 0.1 M phosphate buffer (pH 7.2) at 4°C for 24 hr. After two washes with phosphate buffer, cells were post-fixed with 2% w/v osmium tetra oxide (proSciTech, Thuringowa, Qld, Australia) for 30 min in the same buffer at room temperature

After fixation the cells were washed with phosphate buffer and dehydrated in a graded series of ethanol starting with 70% v/v followed by 80% v/v, 90% v/v and finally 100% v/v. Cells were dried in a critical-point dryer with CO_2 (Polaron, CPD 7501, Quorum, East Grinstead, W. Sussex, UK) with CO_2 and coated with gold (Polaron SC 502 sputter coater, Quorum). The specimens were then examined with a Jeol JSM 6060 scanning electron microscope (Peabody, MA, USA).

Adhesion to Caco-2 cells and viability of bacterial cells *in vitro* under gastrointestinal conditions

All enzymes and chemicals were obtained from Sigma Chemicals. *B. breve* A28, *B. bifidum* A10 and *E. coli* ATCC 11229 were subjected to different treatments in order to observe the effect of various gastrointestinal conditions on their adhesive ability. One ml aliquots of the bacterial suspension were centrifuged (10 000 × *g*, 15 min) and resuspended in 1 ml of artificial gastric juice containing NaCl, 125 mM; KCl, 7 mM; $NaCO_3$, 45 mM; pepsin 3 mg/ml, and HCl to adjust the final pH to 2.0, 3.0, 4.0 and, for a control, 7.0, after which a mixture of simulated pancreatic juice and bile containing 0.15% bile salts, 1 mg/ml of trypsin, and 1 mg/ml of α-chymotrypsin was added (26). All suspensions were incubated for 1 hr at 37°C (except for the cells treated with gastric plus pancreatic juice, which were incubated for two successive hours, one with each treatment), washed, resuspended to their original volume in PBS and combined immediately with epithelial cells in the adhesion reaction mixtures. After incubation of the bacterial suspensions with artificial gastric

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juice and artificial gastric juice plus pancreatic juice, seven successive (10^{-1}–10^{-7}) dilutions were plated on MRSC and nutrient agar to assess the growth of *B. breve* A28, *B. bifidum* A10 and *E. coli* ATCC 11229, respectively. The MRSC agar plates were incubated for 48 hr at 37°C, while nutrient agar plates were incubated for 24 hr at 37°C, then viable cell counts (CFU/ml) were estimated for each plate. In all cases, aliquots of each bacterial suspension, without chemical or enzymatic treatment, and also gastric and pancreatic juices at pH 7, were used as controls. In addition, anaerobic conditions were created by Anaerocult A gas packs (Merck) for all experiments. Subsequently, adherence assays were performed by adding 1 ml of bacterial suspension treated with artificial gastric juice and artificial gastric juice plus pancreatic juice to1 ml of the cell-line growth medium. The adhesion index was calculated.

Statistical analysis

ANOVA followed by Tukey's test was used for data comparison among tested strains. Differences were considered to be significant at $P < 0.05$. Data were analyzed using SPSS 16.0 for Windows statistical software.

RESULTS

EPS production and HA ability of *B. breve* A28 and *B. bifidum* A10

In this study the EPS producing capacity of *B. breve* A28 and *B. bifidum* A10 was determined and the results are shown in Table 1. While *B. breve* A28 had a high EPS producing capacity (97.64 ± 2.00 mg/l), *B. bifidum* A10 has found to have a low EPS producing capacity (38.00 ± 1.00 mg/l). Because the HA ability of bifidobacteria is thought to be useful in preselecting it as having strong adhesion adhesion, this property was also investigated. *B. breve* A28 showed a high degree of HA ability (+3), but *B. bifidum* A10 showed none (0). Table 1 and Figure 1 show the HA abilities of *B. breve* A28 and *B. bifidum* A10.

Table 1. EPS production, HA ability, and inhibitory effect of bifidobacteria

Strain	EPS production (mg/l)*	HA ability	Inhibition zone (mm)**
B. breve A28	97.64 ± 2.00	+3	9 ± 1
B. bifidum A10	38.00 ± 1.00	–	5 ± 1

*Values are mean of values of three independent determinations ± standard errors.

**Inhibition effect of bifidobacteria on *E. coli* ATCC 11229.

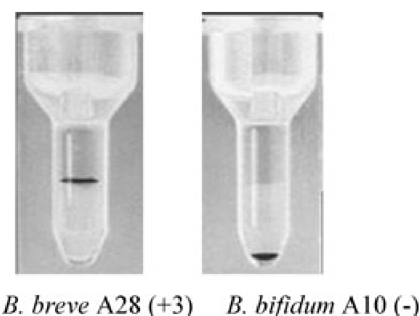

Fig. 1. Shows strong HA ability of *B. breve* A28 (+3) and weak HA ability of *B. bifidum* A10 (–).

The inhibitory effects of *B. breve* A28 and *B. bifidum* A10 on *E. coli* ATCC 11229

Both *B. breve* A28 and *B. bifidum* A10 were determined to be inhibitory to *E. coli* ATCC 11229, which is known as an important intestinal pathogen. While the diameter of inhibition of *E. coli* ATCC 11229 by *B. breve* A28 was 9 ± 1 mm, the diameter of inhibition of *E. coli* ATCC 11229 by *B. bifidum* A10 was found to be 5 ± 1 mm (Table 1).

Adhesion of bacteria to Caco-2 cells and competition

The adhesiveness of *B. breve* A28, *B. bifidum* A10 and *E. coli* ATCC 11229 was quantified by light microscopy Gram staining. Fifty microscopic fields were randomly counted to assess the number of bacteria attached to the epithelial cells. The average numbers of bacteria (± standard deviation) adhering per 100 epithelial cells were calculated from these data and defined as the adhesion index. The adhesive indices of all three strains are summarized in Table 2. The adhesion indices of *B. breve* A28 (300<) and *E. coli* ATCC 11229 (300<) were higher than that of *B. bifidum* A10 (19 ± 1) or control groups. When compared with *E. coli* ATCC 11229, the high EPS-producing *B. breve* A28 showed a better capacity to adhere to Caco-2 cells than did the low EPS-producing *B. bifidum* A10. All these results were confirmed by electron microscopy. When we compared *B. breve* A28 at pH 7 (Figure 2a) with *B. bifidum* A10 at pH 7 (Figure 2b), the high EPS-producing *B. breve* A28 showed better adhesion than did the low EPS-producing *B. bifidum* A10. Moreover, *B. breve* A28, which has a high HA ability, showed strong adhesion capacity. In addition, when the adhesion of *E. coli* in combination with bifidobacteria to Caco-2 cells was investigated, the adhesion ability of *E. coli* was reduced in the presence of bifidobacteria. When the findings were compared, it was found that the effects of competition on *E. coli* ATCC 11229 were greater with the *E. coli* ATCC 11229- *B. breve*

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Table 2. Adhesion of *B. breve* A28 and *B. bifidum* A10 strains with *E. coli* ATCC 11229 under competitive conditions

	Adhesion index[a,b]		
		Competitive conditions	
Strains	Control	A28 and *E. coli*	A10 and *E. coli*
B. breve A28	300<	41 ± 2	
B. bifidum A10	19 ± 1		17 ± 1
E. coli ATCC 11229	300<	48 ± 2	105 ± 8

[a]Adhesion index (number of bacteria attached/100 Caco-2 cells) was evaluated in 50 random microscopic fields and the mean ± standard deviation of adhering bacteria per 100 cells of epithelial cell-line was determined.

[b]Each value of adhesion index represents the average of at least two independent experiments ± mean S.D. of the individual experiments.

A28 combination than with the *E. coli* ATCC 11229- *B. bifidum* A10 combination.

Adhesion to Caco-2 cells and viability of bacterial cells *in vitro* under gastrointestinal conditions

The viability of *B. breve* A28, *B. bifidum* A10 and *E. coli* ATCC 11229 (as a control) was determined under gastrointestinal system conditions, and the results are shown in Table 3. When the effect on viability of changes in pH induced by artificial gastric juice was evaluated, the viable bacterial count was found to decrease for all three bacteria tested, namely *B. breve* A28, *B. bifidum* A10 and *E. coli* ATCC 11229. The greatest inhibitory effected was determined to be at pH 3. In the presence of gastric juice at pH 3, the viability of *B. breve* A28 decreased by about 5.8 log cycles after 1 hr of exposure. *B. bifidum* A10 also lost viability after treatment with gastric juice at pH 3. Taken together, these results suggest that EPS production decreases the effect of artificial gastric juice on the viability of *B. breve* A28. After treatment with artificial gastric plus pancreatic juice, *B. breve* A28, *B. bifidum* A10 and *E. coli* ATCC 11229 were all determined to be more severely affected by the artificial gastric juice alone. *B. breve* A28, *B. bifidum* A10 and *E. coli* ATCC 11229 all lost viability after treatment with gastric juice at pH 3 followed by pancreatic juice. The viability of *E. coli* ATCC 11229 was found to be better than that of *B. breve* A28 and *B. bifidum* A10 after treatment with both artificial gastric juice and gastric plus pancreatic juice.

The effect of gastrointestinal conditions on the adhesion of bacteria to Caco-2 cells was also examined by exposing bacterial suspensions to artificial gastric and gastric plus pancreatic juices, sequentially. The results of these experiments are present in Table 4. In the different gastric juice conditions different adhesion results were found. All these results paralleled the viability results. Correlations between adhesion and viability in different gastric juice conditions were found. Gastric juice at different pH (3, 4, 5 and 7) and gastric plus pancreatic juices treatment all significantly reduced adhesion ($P < 0.05$). After gastric juice treatment, all strains showed better adhesion ability than after gastric plus pancreatic juice treatment. The high EPS-producing *B. breve* A28 showed greater viability and adhesion ability than did the *B. bifidum* A10 after artificial gastric and gastric plus pancreatic juices treatments. These results clearly show that EPS may play protective and adhesion-promoting roles in the intestinal ecosystem. When compared with *E. coli* ATCC 11229, *B. breve* A28

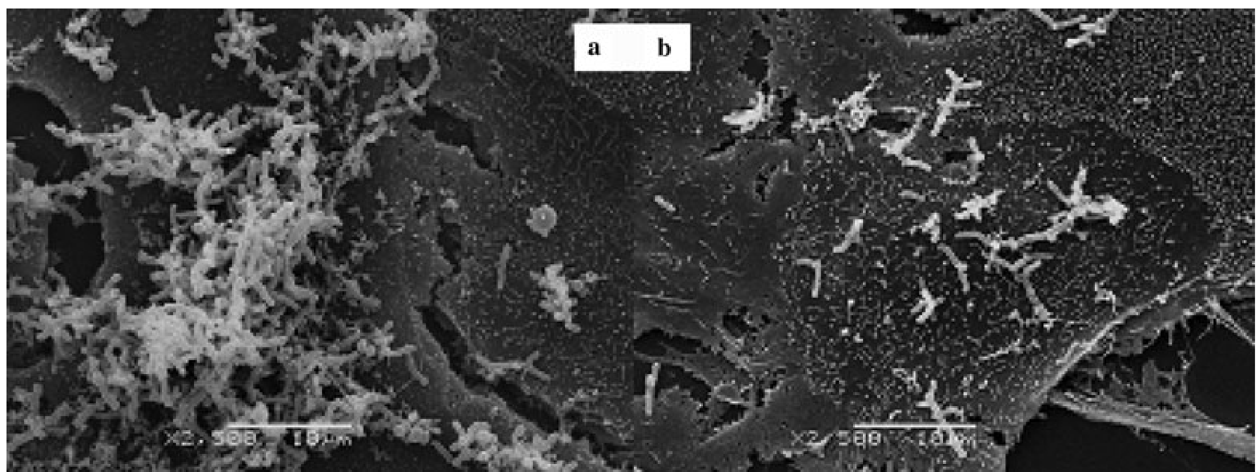

Fig. 2. (a) Adhesion of high EPS producing- *B. breve* A28 and (b) low EPS producing- *B. bifidum* A10 to Caco-2 cells as observed by scanning electron microscopy. (Magnification ×2500).

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Table 3. Viability of bacteria ($\log_{10}$ CFU/ml) after treatment with artificial gastric juice (pH: 3.0, 4.0, 5.0, 7.0) and artificial gastric (pH: 3.0, 4.0, 5.0, 7.0) plus pancreatic juice

	Viability[a,b]							
	Artificial gastric juice*				Artificial gastric plus pancreatic juice*			
Strains	pH 7.0	pH 5.0	pH 4.0	pH 3.0	pH 7.0	pH 5.0	pH 4.0	pH 3.0
B. breve A28	8 ± 1	6 ± 0	5 ± 0	2 ± 0	6 ± 0	6 ± 0	4 ± 1	0
B. bifidum A10	7 ± 2	5 ± 2	3 ± 2	0	5 ± 1	5 ± 0	3 ± 0	0
E. coli ATCC 11229	9 ± 2	9 ± 1	8 ± 2	7 ± 2	9 ± 1	8 ± 1	8 ± 0	7 ± 1

*Mean S.D. of individual experiments.

[a]Viability of bacteria calculated as $\log_{10}$ CFU/ml.

[b]All experiments were performed under anaerobic conditions, which were created by Anaerocult A gas packs (Merck).

was more strongly adhesive than *B. bifidum* A10 under all conditions. While the presence of viable bacteria was not confirmed at pH 3 gastric juice and gastric plus pancreatic juice treatment, these strains did show adhesion to Caco-2 cells.

DISCUSSION

Probiotics can be defined as microbial cell preparations or components of microbial cells that have a beneficial effect on the health and well-being of the host (27). The main criteria for selecting probiotic strains are their acid and bile tolerance, ability to survive throughout the gastrointestinal tract, ability to adhere to intestinal surfaces, temporary colonization, antagonism against pathogens and good technological properties (28). In addition, EPS production by probiotics is important as it enables adhesion to the epithelium and protects the bacterium. EPS production could act as an adherence factor that may play a role in the colonization of intestinal mucosa by probiotics (29). The bacteria that produce EPS can protect themselves from negative factors such as low pH by using EPS like a covering (30, 31). According to our results, the viability of high EPS producing *B. breve* A28 is better than that of low EPS producing *B. bifidum* A10 under gastrointestinal conditions. The protective effects of EPS production are demonstrated by the results presented here. Although several other probiotic bacteria have been reported to produce EPS (32), there are few studies on EPS production by *Bifidobacterium* species. These studies have focused on the structure and characterization of EPS. On the other hand, the HA ability of bacteria may be provide an important adhesin for epithelial surfaces. Ocana *et al*., taking advantage of the similarity between mammalian epithelial cell receptors and erythrocyte membrane receptors, evaluated bacteria for HA ability and concluded that the HA test would be a useful initial step before carrying out the epithelial adhesion test (16). In our study, the HA abilities of bifidobacteria were determined as a selection step for adhesion of bacteria. After an HA ability study, the adhesion index of *B. bifidum* A10 was compared to that of *B. breve* A28. The high HA ability of *B. breve* A28 was found to correlate positively with its adhesion ability, when compared with *B. bifidum* A10.

An important function of the microflora is to exert a barrier against colonization of the gastrointestinal tract

Table 4. Adhesion of bifidobacteria and *E. coli* ATCC 11229 to Caco-2 cells after treatments with artificial gastric juice and artificial gastric plus pancreatic juice

	Adhesion index[a]							
	After treatment with artificial gastric juice*				After treatment with artificial gastric plus pancreatic juice *			
Strains	pH 3	pH 4	pH 5	pH 7	pH 3	pH 4	pH 5	pH 7
B. breve A28	19 ± 2	29 ± 0	42 ± 3	43 ± 1	11 ± 1	17 ± 1	29 ± 1	33 ± 1
B. bifidum A10	6 ± 0	9 ± 1	14 ± 0	19 ± 0	4 ± 1	5 ± 1	8 ± 1	14 ± 0
E. coli ATCC 11230	98 ± 1	112 ± 2	127 ± 2	117 ± 3	93 ± 0	96 ± 0	104 ± 4	115 ± 2

[a]Adhesion index (number of bacteria attached/100 Caco-2 cells) was evaluated in 50 random microscopic fields and the mean ± S.D. of adhering bacteria per 100 cells of epithelial cell-line was determined.

*Asterisks indicate signification differences from their respective controls at different pH values ($P < 0.05$) and mean S.D. of individual experiments.

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by pathogenic bacteria (33). Adhesion of lactobacilli and competitive exclusion of pathogens from intestinal and uroepithelial cells and mucus have recently been well documented. Since bifidobacteria are members of the dominant microflora, it may be more relevant to use this genus as a probiotic, especially in newborns (34). It has been suggested that Bifidobacteria function as an anti-infectious barrier to pathogenic bacteria (35). Increasing their numbers may, therefore, be desirable. In the competitive study reported here, it was observed that *B. bifidum* A10 did not prevent the adhesion of *E. coli* ATCC 11229 to Caco-2 cells as well as *B. breve* A28 did. This difference may be due to the lower EPS production ability of the former strain. A similar study has been reported by Coconnier *et al.* (36). Their studies showed that lactobacilli can exclude enterotoxigenic *E. coli* from Caco-2 cells (36). Moreover, Roselli *et al.*, researched the effects of *B. animalis* MB5 and *L. rhamnosus* GG strains when used as probiotics to protect Caco-2 cell from inflammation caused by enterotoxigenic *E. coli* K88, and observed inhibition of *E. coli* K88 by these bacteria (37).

Adhesion to the intestinal mucosa is considered to be a factor that is important for consequent colonization by the microflora of some areas of the gastrointestinal tract (38). Because bacterial adhesion to epithelial cells has been considered to be one of the selection criteria for probiotic strains, the Caco-2 cell line has been used as an *in vitro* model for intestinal epithelium, this cell line having been used to screen for adhesive strains (39). For these microorganisms, adhesion ability has become an important property in the selection of strains for probiotic purposes. While there are few reports about the adhesion ability of bifidobacteria, researchers have reported their adhesion to Caco-2 cells and mucus (38, 40, 41). In our studies, ability of bifidobacteria to adhere to Caco-2 cells was determined. In addition, in this study the importance of EPS production by bifidobacteria in regard to adhesion to Caco-2 cells has been emphasized. In the present study, scanning electron microscopy revealed that *B. breve* A28 adhered well to Caco-2 cells. Scanning electron microscopic examination also revealed that fibrils emerge from the cell surface of *B. breve* A28, presumably contributing to adhesion to the epithelium (Fig. 2).

Moreover, some studies on adhesion of bifidobacteria to Caco-2 cells have examined them under gastrointestinal conditions. Similarly to our study, Ram and Chander have determined adhesive capacity of *B. bifidum* HI 39 and *B. bifidum* HI 48 strains at different pH (pH 5, 6, 7, 8). Strains were maximally adhesive at pH7 (80–90%), minimum degrees of adhesion to epithelial surfaces being found at pH 5 (15). In our study, both *B. breve* A28 and *B. bifidum* A10 were maximally adhesive to epithelial surface at pH 7. Hood and Zottola observed that exposure of *Lactobacillus acidophilus* BG2FO4 to pH 2, 3, and 4 for up to 5 hr did not affect its ability to adhere to human intestinal tissue cells, however its viability was greatly decreased (42). In our study, adhesion of *B. bifidum* A10 was also significantly reduced after exposure to artificial gastric plus pancreatic juice. These findings may indicate that adhesion of these strains would be reduced after passage through the stomach. It also shows that resistance to low pH is not only important for viability, but also for adhesion.

Adhesion is an important predictor of the ability of bacteria to colonize the gastrointestinal tract. EPS production has an advantage for adhesion by, and protection of, probiotics. Bifidobacteria which have strong adhesion ability and EPS production are more capable of challenging pathogenic bacteria. These results show a potential for adhesive human bifidobacteria to inhibit cell association and cell entry by human pathogens. According to all the above results, EPS production results in adhesion ability even in dead bacterial cells, and EPS may play a role in competition. Based on our findings that adhesion ability is only mildly affected by gastrointestinal system conditions, *B. breve* A28 strain should be considered promising microorganisms for the development of new probiotic products.

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REFERENCES

1. Salminen S., Ouwehand A.C., Isolauri E. (1998) Clinical applications of probiotic bacteria. *Int Dairy J* **8:** 563–72.
2. Gueimonde M., Luis Noriega T.L., Margolles A., De Los Reyes-Gavilan C.G., Salminen S. (2005) Ability of *Bifidobacterium* strains with acquired resistance to bile to adhere to human intestinal mucus. *Int J Food Microbiol* **101:** 341–6.
3. Oda M., Hasegawa H., Komatsu S., Kambe M., Tsuchiya F. (1983) Antitumor polysaccharide from *Lactobacillus* sp. *Agric Biol Chem* **40:** 1623–5.
4. Duco T., Wieruszeski J.M., Fournet B., Carcano B., Ramos P., Loones A. (1990) Structure of an exocellular polysaccharide produced by *Streptococcus thermophilus*. *Carbohydrate Res* **198:** 313–22.
5. Allan W., Linda C.D. (1998) Diet and bacterial colonization: role of probiotics and prebiotics. *J Nutr Biochem* **9:** 668–75.
6. He F., Ouwehand A.C., Isolauri E., Hashimoto H., Benno Y., Salminen S. (2001) Comparison of mucosal adhesion and species identification of bifidobacteria isolated from healthy and allergic infants. *FEMS Immunol Med Microbiol* **30:** 43–7.
7. Bernet M.F., Brassart D., Neesr J.R., Servin A.L. (1994) *Lactobacillus acidophilus* LA1 binds to cultured human intestinal cell lines and inhibits cell attachment and cell invasion by enterovirulent bacteria. *Gut* **35:** 483–9.
8. Jin L.Z., Marquardt R.R., Zhao X. (2000) A strain of *Enterococcus faecium* (18C23) inhibits adhesion of enterotoxigenic *Escherichia*

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coli K88 to porcine small intestine mucus. *Appl Environ Microbiol* **66:** 4200–4.
9. Forestier C., De Champs C., Vatoux C., Joly B. (2001) Probiotic activities of *Lactobacillus casei rhamnosus*: *in vitro* adherence to intestinal cells and antimicrobial properties. *Res Microbiol* **152:** 167–73.
10. Hampson D.J. (1994) Postweaning *Escherichia coli* diarrhea in pigs. In:Gyles C.L., ed. *Escherichia coli in Domestic Animals and Humans*. Wallingford, UK: CAB International, pp. 171–91.
11. Ouwehand A.C., Salminen S., Isolauri E. (2002) Probiotics: an overview of beneficial effects. *Antonie Van Leeuwenhoek* **82:** 279–89.
12. Teitelbaum J.E., Walker W.A. (2002) Nutritional impact of pre and probiotics as protective gastrointestinal organisms. *Annu Rev Nutr* **22:** 107–38.
13. O'Sullivan G.C., Kelly P., O'Halloran S., Collins C., Collins J.K., Dunne C., Shanahan F. (2005) Probiotics: an emerging therapy. *Current Pharmaceutical Design* **11:** 3–10.
14. Canzi E., Guglielmetti S., Mora D., Tamagnini I., Parini C. (2005) Conditions affecting cell surface properties of human intestinal bifidobacteria. *Antonie van Leeuwenhoek* **88:** 207–19.
15. Ram C., Chander H. (2003) Optimization of culture conditions of probiotic bifidobacteria for maximal adhesion to hexadecane. *World J Microbiol Biotechnol* **19**: 407–10.
16. Ocana V.S., Bru E., De Ruiz Holgado A.P., Nader-Macias M.E. (1999) Surface characteristics of lactobacilli isolated from human vagina. *J Gen Appl Microbiol* **45:** 203–12.
17. Andreu A., Stapleton A.E., Fennell C.L., Hillier S.L., Stamm W.E. (1995) Hemagglutination adherence and surface properties of vaginal *lactobacillus* species. *J Infect Dis* **171:** 1237–43.
18. Mukai T., Kaneko S., Ohori H. (1998) Hemagglutination and glycolipid-binding activities of *Lactobacillus reuteri*. *Lett Appl Microbiol* **27:** 130–134.
19. Ofek I., Doyle R.J. (1994) *Bacterial Adhesion to Cell and Tissues*. New York: Chapman and Hall, pp. 94–135.
20. Alp G., Aslim B. (2010) Relationship between the resistance to bile salts and low pH with exopolysaccharide (EPS) production of Bifidobacterium spp. isolated from infants feces and breast milk. *Anaerobe* **16:** 101–105.
21. Frengova G.I., Simova E.D., Beshkova D.M., Simov Z.I. (2000) Production and monomer composition of exopolysaccharides by yogurt starter cultures. *Can J Microbiol* **46:** 1123–7.
22. Dubois M., Gilles K.A., Hamilton J.K., Peters P.A., Smith F. (1956) Colorimetric method for determination of sugars and related substances. *Anal Chem* **28:** 350–6.
23. Torino M.I., Taranto M.P., Sesma F., Font De Valgez G. (2001) Heterofermentative pattern and exopolysaccharide production by *Lactobacillus helveticus* 15807 in response to environmental pH. *J Appl Microbiol* **91:** 846–52.
24. Harmening D.S., Walker P.S. (1999) *Alternative Technologies in Routine Blood Bank Testing. Modern Blood Banking and Transfusion Practices*. Philadelphia: F.A.D avis Company, pp. 545–58.
25. Lievin V., Peiffer I., Hudault S., Rochat F., Brassart D., Neeser J.R., Servin A.L. (2000) *Bifidobacterium* strains from resident infant human gastrointestinal microflora exert antimicrobial activity. *Gut* **47:** 646–52.
26. Zarate G., Perez Chaia A., Gonzalez S., Oliver G. (2000) Viability and β-galactosidase activity of dairy propionibacteria subjected to digestion by artificial gastric and intestinal fluids. *J Food Protection* **63:** 1214–21.
27. Salminen S., Ouwehand A., Benno Y., Lee Y.K. (1999) Probiotics: how should they be defined? *Trends Food Sci Technol* **10:** 107–10.
28. Ouwehand A.C., Tuomola E.M., Tölkkö S., Salminen S. (2001) Assessment of adhesion properties of novel probiotic strains to human intestinal mucus. *Int J Food Microbiol* **64:** 119–26.
29. Raus-Madiedo P., Gueimonde M., Margolles A., De Los Reyes-Gavilan C.G., Salminen S. (2006) Exopolysaccharides produced by probiotic strains modify the adhesion of probiotics and enteropathogens to human intestinal mucus. *J Food Protection* **69:** 2011–5.
30. Cerning J. (1995) Production of exopolysaccharides by lactic acid bacteria and dairy propionobacteria. *Lait* **75:** 463–72.
31. Looijesteijin P.J., Trapet L., De Vries E., Abee T., Hugenholtz J. (2001) Physiological function of exopolysaccharides produced by *Lactococcus lactis*. *Int J Food Microbiol* **64:** 71–80.
32. Cerning J. (1990) Exocellular polysaccharides produced by lactic acid bacteria. *Fems Microbiol Rev* **87:** 113–30.
33. Reid G., McGroarty J.A., Angotti A., Cook R.L. (1988) *Lactobacillus* inhibitor production against *Escherichia coli* and coaggregation ability with uropathogens. *Can J Microbiol* **34:** 344–51.
34. Bernet M.F., Brassart D., Neeser J.R., Servin A. (1993) Adhesion of human bifidobacteria strains to cultured human intestinal epithelial cells and inhibition of enteropathogen-cell interactions. *Appl Environ Microbiol* **59:** 4121–8.
35. Ballongue J. (1998) Bifidobacteria and probiotic action. In:Salminen, von Wright, eds. *Lactic Acid Bacteria. Microbiology and Functional Aspects*. New York: Marcel Dekker, pp. 519–87.
36. Coconnier M.H., Klaenhammer T.R., Kernéis S., Fourniat S., Servin A.L. (1992) Protein-mediated adhesion of *Lactobacillus acidophilus* BG2FO4 on human enterocyte and mucus-secreting cell lines in culture. *Appl Environ Microbiol* **58:** 2034–9.
37. Roselli M., Finamore A., Britti M.S., Mengheri E. (2006) Probiotic bacteria *Bifidobacterium animalis* MB5 and *Lactobacillus rhamnosus* GG protect intestinal Caco-2 cells from the inflammation-associated response induced by enterotoxigenic *Escherichia coli* K88. *Br J Nutr* **95:** 1177–84.
38. Ouwehand A.C., Kirjavainen P.V., Grönlund M., Isoluari E., Salminen S.J. (1999) Adhesion of probiotic microorganisms to intestinal mucus. *Int Dairy J* **9:** 623–30.
39. Tuomola E.M., Ouwehand A.C., Salminen S.J. (1999) The effect of probiotic bacteria on the adhesion of pathogens to human intestinal mucus. *FEMS Immunol Med Microbiol* **26:** 137–42.
40. Jacobsen C.N., Nielsen V.R., Hayford A.E., Moller P.L., Michaelsen K.F., Parregaard A., Sandström B., Tvede M., Jakobsen M. (1999) Screening of probiotic activities of forty-seven strains of *Lactobacillus* spp. by *in vitro* techniques and evaluation of the colonization ability of five selected strains in humans. *Appl Environ Microbiol* **65:** 4949–56.
41. Bianchi M.A., Del Rio D., Pellegrini N., Sansebastiano G., Neviani E., Brighenti F. (2004) A fluorescence-based method for the detection of adhesive properties of lactic acid bacteria to Caco-2 cells. *Lett Appl Microbiol* **39:** 301–5.
42. Hood S.K., Zottola E.A. (1988) Effect of low pH on the ability of *Lactobacillus acidophilus* to survive and adhere to human intestinal cells. *J Food Sci* **53:** 1514–6.

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