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Influence of Gastrointestinal System Conditions on Adhesion of Exopolysaccharide-producing *Lactobacillus delbrueckii* subsp. *bulgaricus* strains to Caco-2 Cells

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ABSTRACT

This study aimed to assess the transit tolerance of potential probiotic dairy Lactobacillus *strains in human uppergastrointestinal tract* in vitro, *and to evaluate the effect of EPS production on the viability and adhesion of these strains. Survival and adhesion of two exopolysaccharide (EPS)-producing* L. delbrueckii *subsp.* bulgaricus *strains (B3 and B2) and* E. coli *ATCC11229 were assessed after the exposure of different pH (gastric juice) and gastric plus pancreatic juice challenges. In the artificial gastric juice (pH 2), both the viability of the strain B3 and B2 was decreased. Artificial juice treatments significantly reduced the adhesion to caco-2 cells (P< 0.05). High EPS-producing B3 survived better in the adverse gastrointestinal conditions and showed better ability of adhesion to Caco-2 cells when assessed for competition with* E. coli *ATCC 11229 compared to low EPS-producing B2. This investigation showed that EPS production could be affected or be involved in the viability, adherence and competition of* L. delbrueckii *subsp.* bulgaricus *strains and support the potential of B3 strain for development of new probiotic products.*

Key words: adhesion, exopolysaccharide, Caco-2 Cells, gastric-pancreatic juices, *Lactobacillus delbrueckii* subsp. *bulgaricus*

INTRODUCTION

Probiotics are benefic microorganisms that may affect the health of the host by improving the intestinal microbial balance and/or by their action on metabolic activities of indigenous flora (Salminen et al., 1998). It is known that to produce many of their health effects, the probiotic microorganisms must be able to survive the transit through the hostile conditions of the gastrointestinal tract, remain at high levels in the intestine, and without being removed by the peristaltic contractions of the gut. In this sense, microorganisms with a short generation time or the ability to adhere to the intestinal mucosa will have a prolonged persistence in the body of the host (Havenaar et al., 1992). Therefore, two desirable properties for probiotic microorganisms are (i) resistance to gastric acidity, bile and pancreatic enzymes and (ii) adhesion ability to mucosal surfaces (Zárate et al., 2002).

Competitive exclusion has also been used in selecting the novel probiotics (Salminen et al., 1996). The mechanisms involved in the bacterial

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adhesion to epithelial cells and mucus have been extensively studied for pathogenic and beneficial microorganisms (Morata de Ambrosini et al., 1999; Ouwehand et al., 1999a). Because it is difficult to investigate the bacterial adherence *in vivo*, adhesion has been studied using the intestinal cell lines of human origin in the culture as *in vitro* models for intestinal epithelium. The Caco-2 cell line spontaneously differentiates under the standard culture conditions and displays typical features of enterocytic differentiation and has been extensively used to study the organization and function of human intestinal cells (Tuomola and Salminen, 1998). Caco-2 cells have already been shown to behave as an ideal model to mimic the host-pathogen interactions in the gut (Bernet et al., 1993).

Multiple features of the cell surface could act as adhesion factors, differing in their nature between even closely related strains. Bacterial components of the cell wall identified as adhesion determinants include acidic mucopolysaccharides (Beachey, 1981), carbohydrate capsule polymers (Wadström et al., 1987), glycoproteins and carbohydrates (Boris et al., 1998) and combinations of carbohydrates and proteinaceous factors of the cell wall surface (Green and Klaenhammer, 1994). However, there are no studies about the influence on the adhesion of exopolysaccharides producing-*Lactobacillus delbrueckii* subsp. *bulgaricus* strains to the intestinal cells.

Exopolysaccharides (EPSs) are exocellular polymers present in the surface of many lactic acid bacteria. A number of strains of *Lactobacillus* and *Bifidobacterium,* the most common probiotics included in the commercial products, are able to produce these biopolymers (Roberts et al., 1995). EPS is thought to protect the microorganisms against the bacteriophages, antibiotics, and toxic compounds (Looijesteijn et al., 2001; Ruas-Madiedo et al., 2002). Another physiological benefit is that EPS is retained longer in the gastrointestinal tract, so that colonization by the probiotic bacteria can be enhanced (Looijesteijn et al., 2001; Kumar andAnad, 1998). In addition, the health benefits of lactic acid bacteria have been attributed to the production of EPS, which has antitumor, antiulcer, immunomodulating, and cholesterol-lowering activity (Ruas-Madiedo et al., 2002; Gill, 1994). Therapeutic role of EPS-producing probiotics were determined on an experimental colitis model in the rats by Şengül et al. (2006). Consequently, EPS producing probiotic cultures can contribute human health by positively affecting the gut microflora.

Dairy *L. delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus* are the microorganisms extensively used as the starter bacteria for the manufacture of yogurt (Radke-Mitchell and Sandine, 1986; Siitonen et al., 1990) but studies on their probiotic properties have only recently begun (Vinderola and Reinheimer, 2003). To date, there are a few reports about the adhesive properties of dairy *L. delbrueckii* subsp. *bulgaricus* strains. They are traditionally not considered to persist as normal in the habitants of the human intestinal tissues and strains of dairy origin are generally used as negative controls in the studies of adhesion (Elo et al., 1991; Fernández et al., 2003). However, recent studies revealed that dairy strains such as *L. lactis* subsp. *lactis* and *L. lactis* subsp. *cremoris* as well as dairy *Lactobacillus* spp. strains can adhere well to Caco 2 cells and mucus (Ouwehand et al., 1999a; Tuomola and Salminen, 1998).

In this study, two *L. delbrueckii* subsp. *bulgaricus* strains were selected among the 26 isolates according to their capacity of EPS production (Aslim et al., 2006). EPS production abilities of these strains were determined in previous works (Aslim et al., 2006 and 2007). The objective of this study was to investigate the effects of gastrointestinal system conditions on high and low EPS producing-*L. delbrueckii* subsp. *bulgaricus* strains in *in vitro* adhesion ability. The competition of *L. delbrueckii* subsp. *bulgaricus* with *Escherichia coli* ATCC 11229 was also determined to understand the effect of EPS production on competition.

MATERIAL AND METHODS

Microorganisms and growth conditions

Two strains of *Lactobacillus delbrueckii* subsp. *bulgaricus* exhibiting the lowest (B2) and highest (B3) EPS production were selected from the 26 strains isolated from the traditional hand-made yogurts (Aslim et al., 2006; Aslim et al., 2007). The strains were classified by their morphological and cultural properties, catalase test, and the API 50 CHL kit™ analyzed by API LAB™ plus software version 4.0 databases (bioMerieux, France). In addition, the strains were confirmed using 16S rDNA sequence analysis. Universal P5 5′ AGAGTTTGATCATGGCTCAG 3′ and P6 5′

CCATGCAGCACCTGTC 3′ were used in PCR amplification of 16S rDNA and sequencing reactions (Tilsala-Timisjarvi and Alatossava, 1993; Ventura et al., 2000). The sequencing process was performed by the METIS-Biotechnology (Ankara, Turkey), and the sequences obtained were searched against The Gen Bank DNA database using the blast function. B3 and B2 strains were activated by three successive transfer every 24 h in the de Man Rogosa-Sharpe broth (MRS, Merck, Germany) before experimental use. *E. coli* ATCC 11229 used as positive control was maintained on Nutrient agar (Oxoid, England) and cultured in Nutrient broth at 37°C for 16 h before the use. Active *L. delbrueckii* subsp. *bulgaricus* and *E. coli* ATCC 11229 strains were grown statically in the broth at 37°C and harvested by centrifugation (10,000 x *g*, 15 min) at late exponential growth phase (18 and 16 h, respectively). Cells were washed twice with 50 mM KH_2PO_2-Na_2HPO_4, containing 8 g/L NaCl and 2 g/L KCl, pH, 7.2 (phosphate buffered saline, PBS), and finally resuspended in the same buffer. Bacterial concentration of *L. delbrueckii* subsp. *bulgaricus* and *E. coli* ATCC 11229 strains was adjusted to (optical density) OD_{600}=0.6 (approximately1 x 10^8 cfu/mL). All the tests were carried out in three independent assays.

Chemical and enzymatic treatment of bacterial cells

All the chemicals and enzymes were obtained from the Sigma Chemical Co. (Sigma, Farma, Ankara, Turkey). To characterize the gastrointestinal system conditions involved in *L. delbrueckii* subsp. *bulgaricus* B3 and B2 strains adhesion bacterial cells were subjected to different treatments prior to adhesion. One milliliter aliquots of the bacterial suspension were centrifuged (10,000 x *g*, 15 min) and resuspended in 1.0 ml of the following treatment solutions: (i) artificial gastric juice containing NaCl, 125 mM; KCl, 7 mM; Na_2CO_3, 45 mM; pepsin 3 mg/mL, and HCl to adjust the final pH to 2.0, 3.0, 4.0 and for control 7.0; (ii) artificial pancreatic juice containing 0.15% bile salts, 1 mg/ml of trypsin, and 1 mg/ml of α-chymotrysin, pH 8.0 and 7.0 (control) (Zárate et al., 2000). Gastric juice followed by exposure to pancreatic juice. All the suspensions were incubated at 37°C for 1 h (except the cells treated with gastric plus pancreatic juices that 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. Seven successive (10^{-1}-10^{-7}) dilutions were plated on MRS and Nutrient agar to assess the growth of *L. delbrueckii* subsp. *bulgaricus* and *E. coli*, respectively. The MRS agar plates were incubated at 37°C for 48 h, while Nutrient agar plates were incubated at 37°C for 24 h in anaerobic conditions. In all the cases, aliquots of each bacterial suspension, without chemical or enzymatic treatment were used as control (pH 7).

Adhesion Assay

The Caco-2 cell line, a human colon adeno carcinoma cells, was obtained from the cell and virus bank of Foot- and Mouth Disease Institute (Ankara, Turkey) of Ministry of Agriculture and Rural Affairs of Turkey. The cells were cultured in Dulbecco's Modified Eagle's Minimal Essential Medium (DMEM, flow) supplemented with 10% (v/v) heat- inactivated fetal calf serum, 2 mM L-glutamine (Sigma, Farma, Ankara, Turkey), 100 unit of penicillin per mililiter and 100 µg/mL streptomycin (Flow). It was cultured in 25 cm^2 cell culture bottles in an incubator with 10% CO_2 / 90% air at 37°C. For adhesion assays Caco-2 monolayers were placed in 24-well tissue culture plates. Cells were seeded at a concentration of 1 x 10^5 cells per well to obtain confluence and maintained for two weeks. The cell culture medium was changed every other day and replaced by fresh DMEM. Caco-2 monolayers were washed twice with 1.0 ml of sterile PBS before the adhesion assay. After final optical density was adjusted to 0.60 ± 0.02 at 600 nm by spectrophotometer (approximately 1 x 10^8 cfu/mL), 1.0 ml of the test bacterial culture was added to 1.0 ml of complete Caco-2. This suspension (2.0 ml) was added to each well of 24-well tissue culture plates and incubated at 37°C in 10% CO_2 / 90% air atmosphere. After incubation for 60 min, the monolayers were washed twice with sterile PBS (pH 7.2) fixed with methanol, gram stained and examined microscopically. Visual counting of the adhered cells was adopted in this study, since it allowed the differentiation of the Gram-positive *Lactobacillus* and Gram-negative *E. coli*. Adherence was evaluated in 50 random microscopic fields and the mean ± standard deviation of adhering bacteria per 100 cells of epithelial cell-line (adhesion index) was

determined. Adhesion index was the number of bacteria attached /100 epithelial cells, mean numbers ±standard deviation.

Competitive adhesion

In the study of the competition for adhesion on Caco-2 cells, after adjusting the final optical density to 0.60 ± 0.02 at 600 nm, *L. delbrueckii* subsp. *bulgaricus* (B3, B2) 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 bacterium were removed by washing with the 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. For scanning electron microscopy, cells were grown on glass cover slips. After the bacterial adhesion assay, the cells were fixed with 3% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) at 4^{o}C for 24 h. After two washes with phosphate buffer, samples dehydrated in a graded series of ethanol starting with 70% (v/v), followed by 80, 90 and finally 100% (v/v). The cells were dried in a critical-point dryer with CO_2 (Polaron, CPD 7501) and coated with gold (Polaron SC 502 sputter coater). The specimens were then examined with a Jeol JSM 6060 scanning electron microscope.

Statistical analysis

One-way analysis of variance (ANOVA) followed by the Tukey's test was used to evaluate the statistical significance of the differences among the tested strains and gastrointestinal system conditions. Significant differences were considered at $P<0.05$. Data were analyzed using the SPSS 11.0 for Windows^{TM} statistical software.

RESULTS

Two *L. delbrueckii* subsp. *bulgaricus* strains were selected among the 26 isolates according to their capacity of EPS production (Aslim et al., 2006). EPS production abilities of these strains were determined in previous works (Aslim et al., 2006; Aslim et al., 2007). *L. delbrueckii* subsp. *bulgaricus* B3 was high EPS-producing strain (211 mg/L) and *L. delbrueckii* subsp. *bulgaricus* B2 was low EPS-producing strain (28 mg/L). In this study, the survival of *L. delbrueckii* subsp. *bulgaricus* strains and *E. coli* ATCC 11229 (control) in the gastrointestinal system conditions was determined as shown in Table 1. When the effect of pH of artificial gastric juice (GJ) was evaluated on the viability, the viable counts decreased for both, *L. delbrueckii* subsp. *bulgaricus* B3 and B2 strains with decreasing pH (Table 1). The highest inhibition effect was observed at pH 2 in artificial gastric juice. When compared with the control, the viability of *L. delbrueckii* subsp. *bulgaricus* B3 decreased about 5.7 log cycles after 1 h of exposure with pH 2.0 gastric juice. On the other hand, *L. delbrueckii* subsp. *bulgaricus* B2 strain lost the viability after treatment with gastric juice at pH 2.0. On the basis of these results, the effect of gastric juice on the viability of *L. delbrueckii* subsp. *bulgaricus* B3 strain could be decreased by EPS production. Both B3 and B2 strains were more affected, after artificial gastric plus pancreatic juice treatment, than only artificial gastric juice (Table 1). Both *L. delbrueckii* subsp. *bulgaricus* B3 and B2 strains lost viability after treatment with gastric juice at pH 2.0 and pancreatic juice (PJ) sequentially. The control strain of *E. coli* ATCC 11229 survived better than *L. delbrueckii* subsp. *bulgaricus* B3 and B2 strains in both gastric and gastric plus pancreatic juice treatment (Table 1).

The effect of gastrointestinal system conditions on the adhesion ability to Caco-2 cells of *L. delbrueckii* subsp. *bulgaricus* and *E. coli* ATCC 11229 (control) strains was also examined by exposing the bacterial suspensions to artificial gastric and gastric plus pancreatic juices, sequentially. The results of these experiments are presented in Table 2. In the different gastric juice conditions (pH 2.0, 3.0, 4.0) different adhesion results were found. All these results paralleled with the viability results. There was correlation between the adhesion and the viability in different gastric juice condition. Gastric juice in different pH (2.0, 3.0, 4.0 and 7.0) and gastric plus pancreatic juices treatment significantly reduced the adhesion ($P< 0.05$). *E. coli* ATCC 11229 showed higher adhesion index in all the treatments. Gastric juice treatment (with pepsin at pH 2.0) decreased the adhesion percentage by approximately 35%, whereas bile salts and pancreatic enzymes such as trypsin and α-chymotrypsin decreased the adhesion only 15%. The adhesion index for the high EPS-producing B3 strain was higher than B2 strain after both

gastric juice treatment and gastric plus pancreatic juice treatment. In the different gastric juice conditions (pH 2.0, 3.0, 4.0), adhesion percentage of the B3 strain was 23-95%, which was decreased from 27 to 88% after gastric plus pancreatic juice treatment. On the other hand, B2 strain showed lower adhesion percentage (16 and 50%) after gastric juice and gastric plus pancreatic juice treatment. The results showed that EPS might have protective effect and adhesion role in the intestinal ecosystem. These results were confirmed by electron microscopy experiments.

Table 1- In vitro survival of bacteria ($\log_{10}$ cfu/mL) after treatment with artificial gastric juice (pH;2.0, 3.0, 4.0, 7.0) and artificial gastric plus pancreatic juice (pH; 8.0 and 7.0).

Strains		Gastric juice	Gastric plus pancreatic juice
B3	**pH2**	2.4±1.8	0.0
	pH3	6.9±0.6	5.8±1.2
	pH4	7.9±0.4	6.3±0.2
	pH7*	8.1±0.2	7.6±0.5
B2	**pH2**	0.0	0.0
	pH3	6.7±1.4	3.2±1.9
	pH4	7.4±1.9	4.6±1.8
	pH7*	7.8±1.7	7.0±1.3
E. coli **ATCC 11229**	**pH2**	4.9±0.9	7.4±1.1
	pH3	7.9±1.9	7.8±1.9
	pH4	8.6±1.5	8.07±1.2
	pH7*	9.5±1.1	8.4±1.5

*gastric and pancreatic juices at pH 7.0, were used as control.

Table 2 - Adhesion index (number of bacteria attached /100 epithelial cells, mean numbers ±standard deviation) of *L. delbrueckii* subsp. *bulgaricus* strains and *E.coli* ATCC 11229 to Caco-2 cells after treatment with gastric juice and gastric plus pancreatic juice.

Strains		After treatment gastric juice	After treatment gastric plus pancreatic juice
B3	**pH2[a]**	10.52±0.61	9.49±0.7
	pH3[b]	35.98±1.1	24.54±0.9
	pH4[c]	43.47±0.3	30.56±5.5
	pH7[d,*]	45.58±1.3	34.66±0.9
B2	**pH2[a]**	4.0±0.8	2.8±0.2
	pH3[b]	6.87±1.5	6.3±1.5
	pH4[c]	10.21±0.4	8.9±1.9
	pH7[d,*]	20.6±0.5	17.7±7.7
E. coli **ATCC 11229**	**pH2[a]**	60.19±3.8	43.36±11.6
	pH3[b]	107.62±6.7	103.9±0.7
	pH4[c]	116.9±5.0	109.8±3.6
	pH7[d,*]	131.4±11.9	124.8±2.9

[a,b, c, d]: the letters indicate significant differences with their respective control and among different pH values ($P< 0.05$).
*gastric and pancreatic juices at pH 7, were used as control.

When B3 strain was treated with artificial juice at pH 2.0 (Figure 1A) and pH 7.0 (Figure 1B) the same showed better adhesion ability compared to B2 strain at pH 2.0 (Figure 1C) and pH 7.0 (Figure 1D). After gastric plus pancreatic juice treatment, high EPS-producing B3 strain exhibited high adhesion ability when compared with B2 strain (Figure 2). Altered morphology resulted in death (Figure 2A).

Competition of *L. delbrueckii* subsp. *bulgaricus* with *E. coli* ATCC 11229 was also determined to understand the effect of EPS production on the competition. *E. coli* ATCC 11229 was chosen for this study as it could allow to understand the competition of an enterobacterium with a lactic acid bacterium which had a higher and lower EPS production capacity. When compared with *E. coli*

ATCC 11229, high EPS-producing *L. delbrueckii* subsp. *bulgaricus* B3 strain showed better adhesion capacity to Caco-2 cells (Figure 3A) than low EPS-producing *L. delbrueckii* subsp. *bulgaricus* B2 strain (Figure 3B). However, *E. coli* showed maximum adhesion capacity in these experiments (Table 3). According to these results, EPS could play a role in competition.

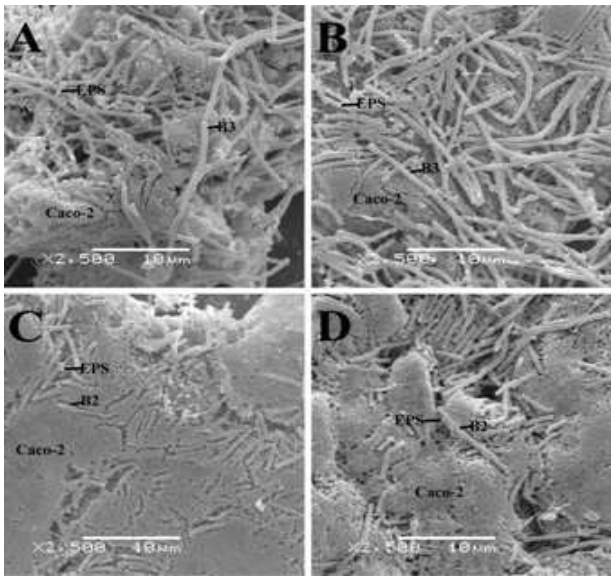

Figure 1 - Scanning electron micrographs of adhesive bacterial strains after treatment with artificial gastric juice at pH 2.0 and 7.0 (GJ-pH 2.0 and GJ-pH 7.0). (A) B3 treated with GJ-pH 2.0, (B) B3 strain with GJ-pH 7.0 (for control), (C) B2 with GJ-pH 2.0, (D) B2 strain with GJ-pH 7.0 (control).

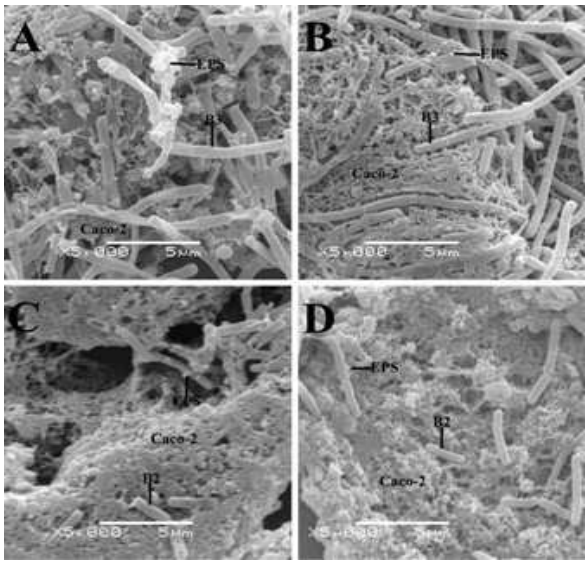

Figure 2 - Scanning electron micrographs of adhesive bacterial strains after treatment with artificial gastric (GJ-pH: 2.0 and GJ-7) plus pancreatic juice (PJ-pH: 8.0), respectively. (A) B3 treated with GJ-pH: 2.0 plus PJ-pH: 8.0 , (B) B3 strain with GJ plus PJ pH:7.0 (for control), (C) B2 with GJ-pH:2.0 plus PJ-pH: 8.0, (D) B2 strain with GJ plus PJ pH:7.0 (for control).

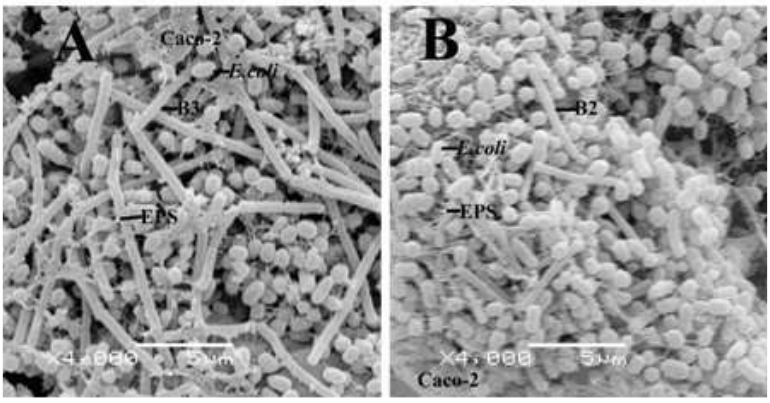

Figure 3 - A: The competition of *E. coli* ATCC 11229 with High EPS producing-B3 which has a higher adhesion capacity. B: The competition of *E. coli* ATCC 11229 with low EPS producing-B2 which has a lower adhesion capacity.

Table 3 - Competitive adhesion index of *L. delbrueckii* subsp. *bulgaricus* B3 and B2 strains with *E. coli* ATCC 11229.

Controls*		**Competition of B3 and *E. coli****		**Competition of B2 and *E. coli****	
E. coli	200.3±1.1	***E. coli***	50.92±3.9	***E. coli***	110.66±12.5
B3	150.7±0.5	**B3**	44.04±2.0	**B2**	20.18±1.0
B2	21.76±0.7				

*All results were given to adhesion index (number of bacteria attached /100 epithelial cells, mean numbers ±standard deviation).

DISCUSSION

EPSs are exocellular polymers present on the surface of many lactic acid bacteria. Some of the EPS-producing strains are currently used in the manufacture of fermented dairy products as a source of natural thickeners and stabilizing ingredients. Positive health effects have been attributed to EPSs (Kumar & Anad, 1998). However, the physiological role that these polymers play in the bacterial ecology of probiotic lactic acid bacteria still remains uncertain. Ruas-Madiedo et al. (2006) reported that the surface characteristics of the probiotic strains could also contribute to the differences in adhesion. The polymers could directly adhere to intestinal mucus and then competitively inhibit the adhesion of the probiotics or they could stick to the probiotic surface and thereby mask the bacterial molecules involved in adhesion.

Bacterial adhesion to intestinal cells and mucus is generally considered as the initial step in the colonization of the gut (Havenaar et al., 1992; Beachey, 1981; Dunne et al., 2001). It has also been related to many of the health effects of probiotic microorganisms, as it prolongs the time that beneficial bacteria can influence the gastrointestinal microbiota and immune system (Ouwehand et al., 1999b). The potential of dairy *L. delbrueckii* subsp. *bulgaricus* as probiotics has been emphasized in recent years (Şengül et al., 2006; Demirer et al., 2006). For these microorganisms, adhesion ability becomes an important property in the selection of strains for probiotic purposes. There are few reports about the adhesion ability of dairy *L. delbrueckii* subsp. *bulgaricus*. Many researchers have reported the adhesion of *L. delbrueckii* subsp. *bulgaricus* to Caco-2 cells and mucus (Ouwehand et al., 1999a; Bianchi et al., 2004; Jacobsen et al., 1999). In the present study, scanning electron microscopy revealed that *L. delbrueckii* subsp. *bulgaricus* B3 adhered well to the Caco-2 cells.

The possibility of probiotic bacteria to colonize the gut depends on their ability to survive the gastric digestion and reach high numbers in the small bowel. However, digestive enzymes and bile present in the gastric and pancreatic secretions (as well as other factors such as peristalsis and competition with indigenous flora) could affect the bacterial adhesion ability. Hood and Zottola (1988) observed that the exposure of *L.*

acidophilus BG2FO4 to pH 2.0, 3.0, and 4.0 up to 5 h did not affect its ability to adhere to human intestinal tissue cells (HITC), but its viability was greatly decreased. In addition, Greene and Klaenhammer (1994) reported that the adhesion of this strain was significantly reduced by the action of pepsin and trypsin but not by chymotrypsin. In the current study, the adhesion of high-EPS producing *L. delbrueckii* subsp. *bulgaricus* B3 was determined after exposure of bacterial suspensions to artificial gastric and gastric plus pancreatic juices sequentially. The viable count of *L. delbrueckii* subsp. *bulgaricus* B3 decreased by 5.7 log cycles at pH 2.0, 1.2 log cycles at pH 3.0, 0.2 log cycles at pH 4.0 in gastric juice as compared with control but *L. delbrueckii* subsp. *bulgaricus* B3 have lost viability in gastric plus pancreatic juice at pH 2.0. The adhesion ability of B3 strain was reduced after gastric juice treatment especially at pH 2.0. Gastric and gastric plus pancreatic juices treatment in different pH (2.0, 3.0, 4.0 and 7.0) significantly reduced the adhesion ($P< 0.05$). On the other hand, low EPS-producing *L. delbrueckii* subsp. *bulgaricus* B2 strain treated with same conditions did not survive in gastric or gastric plus pancreatic juices at pH 2.0. Also this strain showed lower adhesion than *L. delbrueckii* subsp. *bulgaricus* B3 and *E. coli* ATCC 11229 strains after treatment with gastric and gastric plus pancreatic juices at different pH values. *E. coli* ATCC 11229 showed good adhesion capacity after the different exposures to gastric and pancreatic juices. These results showed that the EPS production decreased the effect of gastric and gastric plus pancreatic juices on adhesion ability of *L. delbrueckii* subsp. *bulgaricus* B3. The results pointed to the involvement of the EPSs produced by probiotic strains in their adhesion to caco-2 cells. Scanning electron microscopic examination showed that *L. delbrueckii* subsp. *bulgaricus* B3 presented fibrils that emerged from the cell surface, which could contribute to the adhesion to the epithelium (Figures 1, 2A and B). Zárate et al. (2002) observed same fibrils under the transmission electron microscopy for *Propionibacterium acidopropionici* CRL 1198. This strain showed good adhesion capacity after gastric and pancreatic juice treatments. Gorret et al. (2001) reported EPS production ability of *P. acidopropionici* ATCC 25562 under different pH and temperature conditions. According to these findings, these fibrils could be EPS and this EPS structure could have protective survival role in the gastrointestinal system conditions and adhesion to Caco-2 cells.

Enteropathogenic *E. coli* strains are known to cause gastrointestinal illness in humans and other animals. The attachment of *E. coli* to the mucosa of the small bowel is an early event in the complex colonization process associated with acute diarrheal disease. The adhesion of *E. coli* strains to the intestinal epithelium has been correlated with the production of specific antigens. Cleary et al. (2004) showed the adhesion of the enteropathogenic *E. coli* strains to caco-2 cell line.

Competitive inhibition for the bacterial adhesion sites on intestinal epithelial surfaces is another mechanism of action for the probiotics. The results of competitive binding assays clearly showed that high EPS producing-*L. delbrueckii* subsp. *bulgaricus* B3 reduced the attachment of enteropathogen *E. coli* ATCC 11229 to Caco-2 cells under the condition of exclusion. Coconnier et al. (1992) also observed that lactobacilli excluded the enterotoxigenic *E. coli* from Caco-2 cells. In this competitive study, it was observed that *L. delbrueckii* subsp. *bulgaricus* B2 could reduce the adhesion of *E. coli* ATCC 11229, which decreased from 200.3±1.1 to 110.66±12.5. But this reduction level was not high as B3 strain, which reduced the adhesion of *E. coli* ATCC 11229 from 200.3±1.1 to 50.92 (Table 3). This could be due to the lower EPS production ability of B2 strain. Also, this strain showed lower adhesion index (17.7-21.76) in all the conditions. Under scanning electron microscopic examination, fibrils were observed which were secreted from *L. delbrueckii* subsp. *bulgaricus* B3 strain (Figure 3A).

The results obtained in the present study indicated that the EPSs produced by *L. delbrueckii* subsp. *bulgaricus* B3 strain could be able to adhere to Caco-2 cells. Based on the findings that adhesion ability was slightly affected by the gastrointestinal system conditions, dairy *L. delbrueckii* subsp. *bulgaricus* B3 strain should be considered promising microorganisms for the development of new probiotic products.

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