

Factors Affecting Adhesion of Lactic Acid Bacteria to Caco-2 Cells and Inhibitory Effect on Infection of *Salmonella* Typhimurium

Lim, Sung-Mee^{1*} and Dong-Hyun Ahn²

¹Department of Food Nutrition and Science, Tongmyong University, Busan 608-735, Korea

²Department of Food Science and Technology, Pukyong National University, Busan 608-737, Korea

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In this study, seven strains isolated from mustard leaf *kimchi* were screened for their tolerance to simulated gastric and bile juices, the adhesive properties to Caco-2 cells, and the inhibition ability of *Salmonella* Typhimurium ATCC 29631 adhesion. *Lactobacillus acidophilus* GK20, *Lactobacillus paracasei* GK74, and *Lactobacillus plantarum* GK81, which were resistant to bile as well as gastric juices, possessed high bile-salt hydrolase (BSH) activity towards both sodium glycocholate and sodium taurocholate. The strongest *in vitro* adherence of $53.96 \pm 4.49\%$ was exhibited by *L. plantarum* GK81 followed by *L. acidophilus* GK20 with adhesion levels of $40.72 \pm 9.46\%$. The adhesion of these strains was significantly ($p < 0.05$) reduced after exposure to pepsin and heating for 30 min at 80°C. Addition of Ca^{2+} led to a significant ($p < 0.05$) increase of the adhesion of *L. acidophilus* GK20, but the adhesion ability of *L. plantarum* GK81 was not different from the control by the addition of calcium. In the competition and exclusion experiment, the adhesion inhibition of *S. Typhimurium* by *L. plantarum* GK81 strain was much higher than the other strains. Moreover, the exclusion inhibition of *S. Typhimurium* by *L. acidophilus* GK20 was considerably high, although the inhibition activity of this strain was lower than *L. plantarum* GK81.

Keywords: Adhesion, Caco-2 cells, probiotics, *Salmonella* Typhimurium

Probiotic strains such as *Lactobacillus acidophilus*, *Lactobacillus rhamnosus*, and *Bifidobacterium bifidum* beneficially affect the host by improving intestinal microbial balance and modulating immune responses, and therefore, these microorganisms as biotherapeutic agents have attracted

enormous interest in recent years [8]. Probiotic lactic acid bacteria (LAB), which can alleviate gastrointestinal diseases, prevent infection of pathogenic bacteria, and degrade carcinogens in the gut, have been used in the manufacture of fermented dairy and pharmaceutical products and functional foods [28]. Because of the health-promoting properties of probiotics, the worldwide market of yoghurts and other milk products fermented using probiotics have increased dramatically in recent years [30].

To meet main criteria for the selection of potential probiotic strains and crucial factors for beneficial health effects, probiotic bacteria have to be able to survive under digestive enzymes and gastric juice in the stomach as well as bile secreted in the duodenum [7]. Thereafter, probiotics adhered and colonized to the epithelial cells in the gastrointestinal tract may modulate the indigenous microbes, enhance the intestinal epithelial barrier, stimulate the host immune system, or deactivate virulence factors [27]. Recent studies have explained that probiotic LAB adhered to the intestinal cells are effective to prevent the infection of enteropathogens at an early stage by inhibiting the adhesion of pathogenic bacteria by competition for nutrients and attachment sites or by secretion of antimicrobial substances such as organic acids, bacteriocin, and hydrogen peroxide [11]. Probiotic strains have been found to inhibit the infection of *Salmonella* ssp. to the intestinal epithelial cells [5]. *L. acidophilus* strain LB exhibited strong inhibition activity against both the cell association and the invasion of Caco-2 cells by enteropathogenic *Escherichia coli* and *Listeria monocytogenes* [31].

The adhesion capacity and property of probiotics to human intestinal cells have been reported to be strain specific. *L. acidophilus* BG2FO4 and *Lactobacillus casei* subsp. *rhamnosus* Lcr35 have been shown to adhere to the enterocyte-like Caco-2 cells. Heat-killed *L. acidophilus* LB was also found to adhere as efficiently as the live strains [31]. Furthermore, some *Bifidobacterium* strains have the

*Corresponding author

Phone: +82-51-629-1714; Fax: +82-51-629-1309;
E-mail: limsm020@tu.ac.kr

capacity to adhere to the intestinal cells and the ability to elicit beneficial effects *in vivo* [37]. The ability of probiotic strains to bind could be altered by such culture factors as medium, temperature, and pH [34]. Furthermore, heating, enzymes, and calcium ions have an influence on the bacterial attachment and are involved in specific adhesive interactions with protein and polysaccharide adhesion molecules at the cell surface [38]. Tuomola *et al.* [33] have demonstrated that physical, chemical, and enzymatic pretreatments of probiotic lactobacilli alter their adhesion to human intestinal mucus glycoproteins.

In our previous studies, we confirmed that *L. acidophilus* GK20, *Lactobacillus brevis* GK55, *Lactobacillus paracasei* GK74, *Lactobacillus plantarum* GK81, *Leuconostoc mesenteroides* GK104, *L. brevis* MLK27, and *Pediococcus pentosaceus* MLK67 isolated from mustard leaf kimchi have antibacterial and antioxidant activities or cholesterol-lowering effects [16–18]. Thus, to evaluate the potentiality of these strains as probiotics, we investigated the resistance during passage through the harsh environment of simulated intestinal juices and the adhesion ability to Caco-2 cells of the selected strains in the present study. The effects of physical, chemical, and enzymatic pretreatments on the adhesion of the strains were also assessed. In addition, the inhibitory effects of the adhesion of *Salmonella* Typhimurium ATCC 29631 to the epithelial cell lines by the selected LAB were determined.

MATERIALS AND METHODS

Culture Conditions of LAB and Caco-2 Cells

The seven strains of *L. acidophilus* GK20, *L. brevis* GK55, *L. paracasei* GK74, *L. plantarum* GK81, *Leu. mesenteroides* GK104, *L. brevis* MLK27, and *P. pentosaceus* MLK67 isolated from mustard leaf kimchi were grown in Lactobacilli MRS broth (Difco Co., Sparks, MD, USA) at 37°C and maintained in MRS broth with 20% (v/v) glycerol at –80°C. *S. Typhimurium* ATCC 29631 obtained from the American Type Culture Collection (ATCC) was grown in Brain Heart Infusion (BHI; Difco Co.) broth at 37°C and kept in a deep freezer until just before use. The intestinal Caco-2 cell line obtained from the Korean Cell Line Bank (KCLB) was prepared in Dulbecco's modified Eagle's medium (DMEM; GIBCO, Invitrogen Ltd., Carlsbad, CA, USA) containing 2 mM L-glutamine, 1 mM sodium pyruvate, 10% (v/v) heat-inactivated fetal bovine serum for 30 min at 56°C (FBS; GIBCO), 100 units/ml penicillin, and 50 µg/ml streptomycin, and incubated at 37°C under 5% CO₂–95% air atmosphere. To produce the monolayer of Caco-2 cells, the culture medium was changed every other day during two weeks.

Transit Resistance to Simulated Gastric and Bile Juices

To determine the transit resistance through human gastrointestinal juice, overnight cultures of the LAB were centrifuged (7,000 ×g, 20 min, 4°C), washed twice in phosphate buffer saline (PBS, pH 6.5), and added to a final concentration of 1.0 × 10⁸ CFU/ml in simulated gastric juice (pH 2.0) containing 0.3% (w/v) pepsin (Sigma-

Aldrich, St. Louis, MO, USA), 125 mM NaCl, 7 mM KCl, and 45 mM NaHCO₃. After incubation for 2 h at 37°C, viable cell counts of the LAB were estimated on Lactobacilli MRS agar by the pour plate method. Immediately after incubation under gastric juice, the cultures were resuspended in simulated bile juice consisting of 1 mg/ml pancreatine (Sigma-Aldrich) and 0.5% (w/v) bovine bile (Sigma-Aldrich) and incubated for 3 h at 37°C. Viable cell counts were performed as mentioned above. Inhibition of the LAB under simulated gastric and bile juices was conducted in triplicate and expressed as the percentage of the cell counts reduced or increased from the initial cells.

Bile-Salt Hydrolase (BSH) Assay

The BSH assay was carried out by determining the amount of liberated amino acids from the conjugated bile salts according to the ninhydrin method. Briefly, the strain cells were grown overnight in MRS broth and centrifuged at 10,000 ×g for 10 min at 4°C. The cell pellet was washed twice in 0.1 M PBS (pH 6.5) and resuspended in the same buffer to adjust to a final optical absorbance of approximately 2.0 at 600 nm. For obtaining the cell-free extract, the cell suspensions were sonicated for 3 min with constant cooling in ice water using a sonicator (Q125; Qsonica, USA), followed by centrifugation (10,000 ×g, 4°C, 20 min) to remove the cell debris. The supernatant (0.1 ml) obtained was added to the reaction mixture (1.9 ml) consisting of 6 mM conjugated bile salt (sodium glycocholate or sodium taurocholate; Sigma-Aldrich) dissolved in 0.1 M PBS. After incubation for 30 min at 37°C, the sample was immediately mixed with 0.5 ml of trichloroacetic acid [15% (w/v)] to terminate the reaction and centrifuged (10,000 ×g, 10 min, 4°C) to remove the precipitation. The supernatant (0.2 ml) was thoroughly vortexed with 1 ml of deionized water and 1 ml of ninhydrin reagent [0.2 ml of 0.5 M citrate buffer (pH 5.5), 1.2 ml of 30% glycerol, and 0.5 ml of 1% ninhydrin (Sigma-Aldrich) in 0.5 M citrate buffer] and boiled for 14 min at 100°C. To calculate the concentration of amino acids released, the absorbance of the cooled sample was recorded at 570 nm using glycine or taurine as standards. One unit of BSH activity was defined as the amount of enzyme that releases 1 µM of amino acid from the substrate per minute.

In Vitro Adhesion Assay

For adhesion assay experiments, the monolayers of Caco-2 routinely grown until post-confluence stage in a 12-well culture plate (Falcon, Beckton Dickinson, Sparks, MD, USA) were centrifuged (2,000 ×g, 3 min), carefully washed twice with PBS (pH 6.5), and preincubated in the culture medium without antibiotics for 30 min before addition of the bacterial cells. The LAB cells grown overnight in MRS medium were centrifuged (7,000 ×g, 20 min, 4°C), washed twice in PBS (pH 6.5), and diluted in nonsupplemented DMEM to achieve a concentration of 1.0 × 10⁸ CFU/ml. Subsequently, the bacterial DMEM suspension was transferred onto the monolayers of Caco-2 cells and incubated for 2 h at 37°C under 5% CO₂–95% air atmosphere. After incubation, the bacterial suspension was aspirated and the Caco-2 monolayers were washed five times with PBS to remove the non-adherent bacteria prior to adding trypsin (0.1%)–EDTA (0.53 mM) to detach the adherent bacterial cells. The cells of adhered LAB to Caco-2 cells were determined by counting the colonies formed after aerobic incubation for 48 h at 37°C on MRS agar plates. Simultaneously, the bacteria cells adhered to Caco-2 monolayers were washed five times with PBS, fixed with 2%

formalin for overnight, and stained with 2% eosin Y. Then the cells were washed three times with 1% acetic acid added in 50% ethyl alcohol, and the absorbance was measured at 570 nm using a microplate reader (Packard Instruments, Meriden, CT, USA). Each adhesion experiment was performed in triplicate. Adhesion of the LAB to Caco-2 cell lines was expressed as a percentage of the absorbance ($A_{570\text{ nm}}$) compared with the control.

Determination of Cell Surface Hydrophobicity

Cell surface hydrophobicity assays were conducted according to the ability of the LAB to adhere to hydrocarbons. The cultures of LAB were harvested in the stationary phase by centrifugation at $7,000 \times g$ for 20 min at 4°C , washed twice in PBS (pH 6.5), and finally resuspended in the same buffer. The cell suspension (1 ml) was adjusted to the absorbance ($A_{570\text{ nm}}$) value of approximately 1.0, put in contact with *n*-hexadecane (6 ml), and thoroughly mixed for 2 min using a vortex, in order to form an emulsion. The two phases were allowed to separate at room temperature for 30 min and then the aqueous phase was carefully removed and the absorbance measured at 600 nm using a spectrophotometer. Cell surface hydrophobicity (H%) was calculated with the formula $H\% = [(A_0 - A)/A_0] \times 100$, where A_0 and A are the absorbance (OD_{600}) before and after extraction with *n*-hexadecane, respectively.

Factor Affecting Adhesion Capacity

The bacteria cells were collected by centrifugation ($7,000 \times g$, 20 min, 4°C) and washed twice with PBS (pH 6.5) before they were resuspended in the same buffer at a concentration of approximately 10^8 cells/ml. The suspension was subjected to the following treatments: (1) The cells were thoroughly mixed in an equal volume of PBS adjusted to pH 2.0 (acidic), pH 7.0 (neutral), and pH 11.0 (alkaline). (2) In heating treatments, the bacterial cells were heated for 60 min at 60°C , for 30 min at 80°C , and for 15 min at 100°C . (3) The bacterial cells were mixed with the following proteolytic enzyme solution at a final concentration of 2 mg/ml: trypsin dissolved in 50 mM Tris-HCl buffer (pH 8.0), pepsin dissolved in 10 mM citrate (pH 2.0), and neutral protease dissolved in 50 mM Tris-HCl buffer (pH 7.5), respectively. After incubation for 1 h at 37°C , the enzymes were inactivated by heating (for 5 min at 80°C) prior to adherence assays. (4) The bacteria were added to 0.01 M metaperiodate (Sigma-Aldrich) in 0.1 M citrate-phosphate buffer (pH 4.5). (5) To determine the influence of divalent ions on adhesion of the LAB, the bacterial suspensions were thoroughly mixed in an equal volume of PBS buffer (pH 6.5) containing 10 mM of CaCl_2 and MgCl_2 , respectively. Controls consisted of pH unadjusted bacteria, non-heated bacteria, or bacteria incubated in the buffers without proteolytic enzymes, metaperiodate, or divalent ions. Then the pretreated bacterial suspensions were added to each well containing Caco-2 monolayers in DMEM and incubated for 2 h at 37°C under 5% CO_2 -95% air atmosphere. Thereafter, the bacterial suspensions were discarded and Caco-2 monolayers were washed three times with PBS to remove the non-attached bacteria. Adhesion was measured as the absorbance at 570 nm according to the procedure described above. The experiment was performed in triplicate.

Adhesion Inhibition of *S. Typhimurium* to Caco-2 Cells

S. Typhimurium ATCC 29631 proliferated overnight at 37°C in BHI broth and the LAB incubated in MRS broth were separately harvested by centrifugation ($7,000 \times g$, 20 min, 4°C) and washed twice

with PBS (pH 6.5). For exclusion assay, the LAB were inoculated at a concentration of 1.0×10^8 CFU/ml in antibiotics-free DMEM containing Caco-2 monolayers to 24-well tissue culture plates, preincubated for 1 h at 37°C in 5% CO_2 -95% air, infected with *S. Typhimurium* ATCC 29631 at the same cell counts, and then incubated for 1 h at 37°C under the same gas composition. Additionally, the ability of the LAB to inhibit competitive adhesion of *S. Typhimurium* ATCC 29631 to Caco-2 cells was evaluated by culturing for 2 h at 37°C in 5% CO_2 -95% air atmosphere after simultaneous inoculation of 1.0×10^8 CFU/well of the LAB and the pathogen in antibiotics-free DMEM. Finally, in the displacement assay, the cells (1.0×10^8 CFU/well) of the pathogen in DMEM without antibiotics were added onto Caco-2 monolayers, preincubated for 1 h at 37°C , inoculated with the LAB at the same cell counts with the pathogen, and then incubated for 1 h at 37°C in 5% CO_2 -95% air. After detachment of the cells from the well plates with trypsin-EDTA, serial dilutions of the pathogenic strain were incubated on SS agar (Difco Co.) plates for 24 h at 37°C under aerobic conditions. Inhibition of the pathogen adhesion was quantified by comparison of the counts of the adhered bacteria in the control experiments without the LAB. Each assay was performed in three independent experiments.

Statistical Analysis

All experiments are expressed as the average \pm standard deviation (SD) of three independent experiments and calculated using one-way analysis of variance (ANOVA) provided by the SPSS 12.0 program (SPSS Inc., Chicago, IL, USA). A Duncan's test was used to determine the statistically significant difference ($p < 0.05$) between the means.

RESULTS AND DISCUSSION

Transit Resistance to Simulated Gastric and Bile Juices

Resistance to gastric acid secreted in the stomach and bile in the small intestine is frequently considered as an essential criterion for selection of the potential probiotic

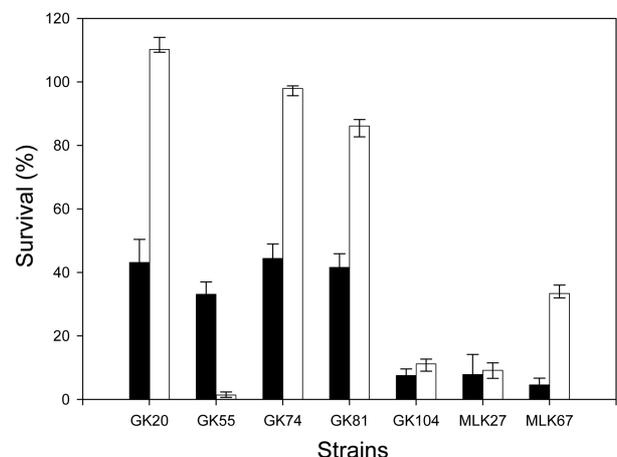


Fig. 1. Survival of the LAB tested after incubation in simulated gastric (■) and bile juices (□). Results are expressed as means of three independent experiments and error bars represent standard deviations.

strain [7]. Thus, the results of resistance to simulated gastric and bile juices of the LAB isolated from mustard leaf *kimchi* are shown in Fig. 1. Viable cell counts of *Leu. mesenteroides* GK104, *L. brevis* MLK27, and *P. pentosaceus* MLK67 after exposure to simulated gastric juice containing pepsin at pH 2.0 were decreased by more than 90% from initial counts within 2 h. However, *L. acidophilus* GK20, *L. brevis* GK55, *L. paracasei* GK74, and *L. plantarum* GK81 showed relatively high resistance to simulated gastric juice. Meanwhile, the exposure of *L. brevis* GK55, *Leu. mesenteroides* GK104, and *L. brevis* MLK27 to simulated bile juice resulted in a rapid loss of the viability. On the other hand, the strains of *L. paracasei* GK74 and *L. plantarum* GK81 showed a high level of survival above 80% after 3 h of incubation. In particular, *L. acidophilus* GK20 proved to be quite resistant even in the presence of 0.5% bile, since viable numbers of this strain after 3 h were increased about 10% over initial counts. Therefore, the viability of *L. acidophilus* GK20, *L. paracasei* GK74, and *L. plantarum* GK81 was retained highly under simulated gastric and bile juices, so the residual cells of these strains after passing through intestinal juices may be sufficient to adhere to and colonize epithelial cells.

Schillinger *et al.* [30] reported that under an *in vitro* system simulating gastric transit, the strains of the *L. acidophilus* group were tolerant to acid conditions at pH 2.0, but the viability of *L. paracasei* strain under pepsin-containing simulated gastric juice was lost completely within 30 min. Vinderola and Reinheimer [36] demonstrated that among the probiotic bacteria tested, *L. acidophilus* showed high values of resistance to gastric and bile juices.

Generally, acids are able to enter their undissociated form in the cell membrane *via* passive diffusion, release hydrogen ions in the cytoplasm, and then finally cause cell death [25]. However, the main mechanism of the acid tolerance in some strains such as LAB involves the multi-subunit F_1F_0 -ATPase, which pumps excess protons from the cytoplasm at the expense of ATP and consequently induces intracellular pH homeostasis [20]. Meanwhile, the survival of the strains in gastrointestinal fluid is obviously improved by the buffering capacity of food components, which may provide a protective matrix enhancing survival of the strains [23]. The probiotic bacteria certainly have to survive in gastric juice of the stomach and bile juice of the intestinal tract after ingestion, in order to exert their beneficial effects after arrival in the intestine.

BSH Activity

All the strains showed varying degrees of BSH activity towards both sodium glycocholate and sodium taurocholate (Fig. 2). More glycine-conjugated bile salt was found to be efficiently deconjugated by the strains of *L. plantarum* GK81, *L. brevis* MLK27, and *P. pentosaceus* MLK67

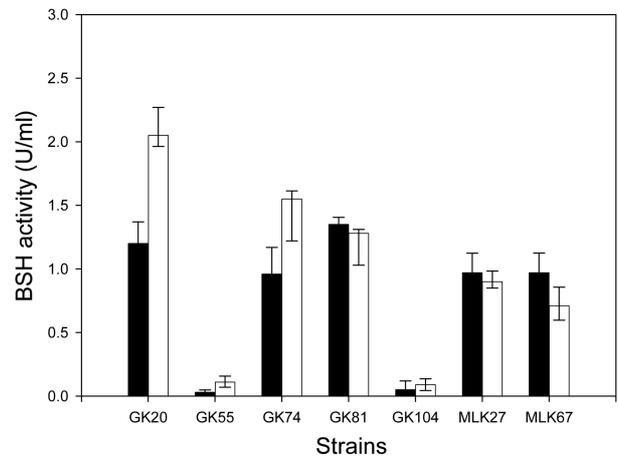


Fig. 2. BSH activity against sodium glycocholate (■) and sodium taurocholate (□) of the tested strains. Results are expressed as means of three independent experiments and error bars represent standard deviations.

than taurine-conjugated bile salt. However, *L. acidophilus* GK20 and *L. paracasei* GK74 showed substrate preference towards sodium taurocholate compared with sodium glycocholate. Among the LAB tested, *L. plantarum* GK81 and *L. acidophilus* GK20 showed the highest total BSH activity towards glycine- and taurine-conjugated bile, respectively, whereas *L. brevis* GK55 and *Leu. mesenteroides* GK104 exhibited the lowest total BSH activity towards sodium glycocholate and sodium taurocholate, respectively. These results suggested that *L. acidophilus* GK20, *L. paracasei* GK74, and *L. plantarum* GK81, which were resistant to bile juice among the seven LAB tested, possessed high BSH activity.

Bile acids, cholesterol metabolites have been classified in two groups; primary bile acids (mainly chenodeoxycholic acid and cholic acid) are synthesized from cholesterol in the liver and secondary bile acids (lithocholic acid and deoxycholic acid) are produced by intestinal bacteria in the distal ileum and colon [1]. In liver, they are produced as bile salts that are glycol- and tauro-conjugated after being peptide-bound with glycine or taurine and then self-assembled into micelles. Bile salts can alter conformation of the cell membrane proteins and plug into the membrane lipids, which may change the integrity and permeability of the membrane by protein misfolding or denaturation. In addition, they are known to be responsible for generating free radicals and damaging or altering the structure and function of nucleic acids [15]. BSH, which hydrolyzes the peptide bond of conjugated bile acids and contributes to the protection mechanisms against toxicity of the conjugated bile salts in the duodenum, is commonly expressed by gastrointestinal bacteria and probiotics such as *Bacteroides* spp., *Bifidobacterium* spp., *Clostridium* spp., *L. acidophilus*, *Lactobacillus gasseri*, *Lactobacillus johnsonii*, and *L.*

plantarum [2, 6]. BSH that induces transformation of the fluidity, potential, and tensile strength of the bacterial membrane affects the bile salt tolerances of the bacteria and improves the viability of enterobacteria in the intestinal tract. In addition, the enzyme that promotes the incorporation and the precipitation of cholesterol into the cellular membranes of the bacteria results in the cholesterol lowering effects [15].

Interestingly, bile tolerance and BSH activity are of a strain-specific nature. Schillinger *et al.* [30] demonstrated that among the lactobacilli strains isolated from probiotic dairy products, BSH activity was detected in all the strains of *L. acidophilus* and *L. johnsonii* with the exception of *Lactobacillus casei* group. Liong and Shah [19] found that among 11 strains of lactobacilli, *L. acidophilus* ATCC 33200, 4356, and 4962 and *L. casei* ASCC 1521 showed the highest BSH activity towards bile mixtures that resemble human bile, and may be promising candidates to exert beneficial bile deconjugation activity *in vivo*.

In Vitro Adhesion Activity

As shown in Fig. 3, the *in vitro* adhesion level to Caco-2 cells was highly strain-dependent. The strongest *in vitro* adherence of $53.96 \pm 4.49\%$ was exhibited by *L. plantarum* GK81, followed by *L. acidophilus* GK20 with adhesion levels of $40.72 \pm 9.46\%$. At this time, the cell counts of *L. plantarum* GK81 and *L. acidophilus* GK20 binding to Caco-2 cells were estimated at 4.97 ± 0.50 log CFU/well and 3.14 ± 0.66 log CFU/well, respectively. Viable cell counts of *L. paracasei* GK74 and *L. brevis* MLK27 adhered to Caco-2 cells were 2.44 ± 0.56 log CFU/well and 2.19 ± 0.73 log CFU/well, respectively. However, *L. brevis* GK55 and *Leu. mesenteroides* GK104 had poor adhesion capacity, because the cell counts of both the strains adhered to Caco-2 cells were even below 1 log CFU/well.

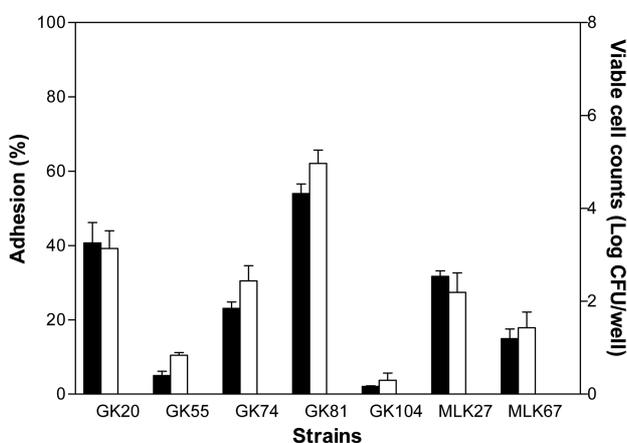


Fig. 3. Adhesion rate (■) and cell counts (□) of the LAB adhered to Caco-2 cells.

Results are expressed as means of three independent experiments and error bars represent standard deviations.

The important adhesion factors of probiotic strains to the epithelial cells are involved in mannose-specific adhesion and adhesion-promoting proteins located on the cell surface [35]. Kos *et al.* [13] demonstrate that the relationship between the autoaggregation and the adhesiveness ability of *L. acidophilus* M92 was mediated by proteinaceous components on the cell surface. Morata *et al.* [21] suggested that the presence of lectin-like substances on the cell wall of *L. casei* CRL 431 is important to the adhesion phenomenon, since the adherence capacity was lost after removal of these substances. In addition, the process of the adhesion includes electrostatic and hydrophobic interactions, steric or passive forces, lipoteichoic acids, exopolysaccharides, and specific bacterial structures such as external appendages, which contribute to specific and/or non-specific adhesion [31].

The strains adhered and colonized to the gastrointestinal tract may enhance the capacity to stimulate the immune system and heal the damaged intestinal tissue, so the adhesion of strains to the intestinal mucosa was considered one of the main selection criteria for a probiotic microorganism with beneficial health effects [7]. Some authors indicated that the adhesion and colonization of probiotic LAB to the intestinal mucosa may be important for alleviating specific gastrointestinal diseases such as ulcerative colitis and rotavirus diarrhea and reducing the colonization of the pathogens [23]. Jensen *et al.* [12] revealed the major species or strain differences and matrix distinction for the adhesion properties and mechanism of probiotic bacteria. Circumstances such as the immune systems of hosts, competition with the intestinal bacterial flora, mucosal shedding, and peristaltic flow in the human gastrointestinal tract are likely to modify the bacterial adhesion.

Cell Surface Hydrophobicity

In the five strains showing relatively high adhesion, considerable differences of cell surface hydrophobicity between the strains were observed in the range from $0.58 \pm 0.16\%$ (*P. pentosaceus* MLK67) to $19.18 \pm 1.60\%$ (*L. plantarum* GK81) (Fig. 4). According to the hydrophobicity and adherence values, it was possible to conclude that the hydrophobicity and the adhesion ability of *L. plantarum* GK81 and *L. brevis* MLK27 had some kind of correlation. Although the strain of *L. acidophilus* GK20 had a strong adhesion property to Caco-2 cells, the surface hydrophobicity of this strain was much lower than that of *L. brevis* MLK27. These are in agreement with observations reported by Ouwehand *et al.* [24], who did not observe any correlation between the surface hydrophobicity and the adhesion of the probiotic microorganisms to intestinal mucus. In addition, *L. bulgaricus* and *Lactococcus lactis* ssp. *cremoris* were found to be tightly attachment to the immobilized mucus, but there was no correlation between the cell surface hydrophobicity and the adhesion ability of

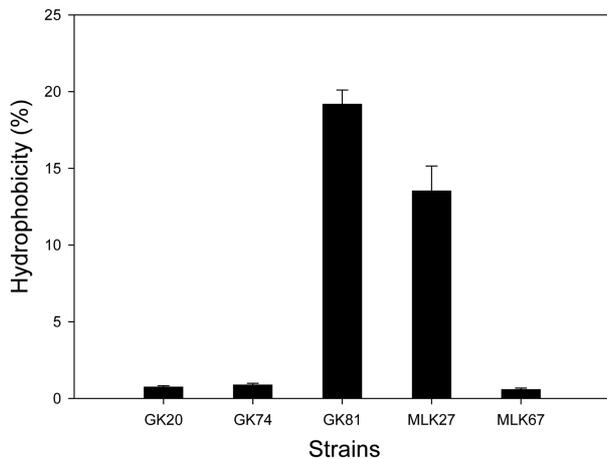


Fig. 4. Cell surface hydrophobicity of the five LAB showing high adhesion ability.

Results are expressed as means of three independent experiments and error bars represent standard deviations.

the strains. Conversely, a direct correlation was found between the bacterial hydrophobicity and the adhesion of the three *Lactobacillus* strains to uroepithelial cells, and significant differences of the hydrophobicity for each type of strain might exist owing to the composition and the structure of bacterial surface [26]. High surface hydrophobicity of the strains may play an important role in adhesion and colonization of the bacteria to epithelial cells and facilitate the contact between microorganism and intestinal cell of the host, although it is obviously not a prerequisite for an adherence capacity of the strains [30]. More detailed investigations on the cell wall of *L. johnsonii*, *Lactobacillus crispatus*, and *Lactobacillus helveticus* strains demonstrated

the enormous variation in hydrophobic/hydrophilic properties among closely related species and even strains of the same species [29].

Factors Affecting the Adhesion Capacity

The effects of various factors such as pH, heating, oxgall, metaperiodate, enzymes, and divalent ions on the adhesion of the five strains are shown in Table 1. Treatment of the bacteria at acidic, neutral, and alkaline pH did not significantly ($p < 0.05$) influence the adherence of *L. plantarum* GK81 and *L. brevis* MLK27 strains, indicating that the bacterial cell structures needed for the adhesion were not irreversibly affected at extreme pH values. However, the adhesion of *L. acidophilus* GK20, *L. paracasei* GK74, and *P. pentosaceus* MLK67 was significantly ($p < 0.05$) reduced after exposure to pH 2.0, although their adhesion did not change at neutral and alkaline pH. These findings may indicate that the resistance to low pH is not only important for the survival but also for the adhesion. Granato *et al.* [9] demonstrated that although pH may play an important role in enhancing the adhesion of lactobacilli, it is not the sole factor involved. These results are in accordance with a previous report, which indicated that low pH treatment reduced the adhesion of all lactobacilli strains except *L. rhamnosus* LC-705 [23].

In the case of heat treatment for 60 min at 60°C, the adhesion of all strains was maintained at similar levels with the control. When heating for 30 min at 80°C, the adhesion ability of *L. acidophilus* GK20, *L. paracasei* GK74, and *L. plantarum* GK81 strains to Caco-2 cells was considerably decreased, whereas *L. brevis* MLK27 and *P. pentosaceus* MLK67 had no effect on their ability to adhere to Caco-2 cells *in vitro*. Because the adhesion

Table 1. Effects of physical, chemical, and enzymatic treatments of the LAB tested on the adhesion to Caco-2 cells.

Treatments	Adhesion (%)				
	GK20	GK74	GK81	MLK27	MLK67
Control	40.72 ± 9.46	23.07 ± 3.01	53.96 ± 4.49	31.73 ± 2.51	14.97 ± 4.48
pH 2.0	8.60 ± 3.70*	3.26 ± 1.34*	55.78 ± 2.98	34.41 ± 6.77	4.71 ± 0.98*
pH 7.0	37.01 ± 3.51	25.38 ± 9.20	51.28 ± 3.70	30.66 ± 3.50	16.70 ± 2.89
pH 11.0	38.00 ± 3.81	22.82 ± 3.38	50.41 ± 1.97	32.88 ± 2.30	15.27 ± 1.76
Heating (60°C, 60 min)	38.79 ± 5.39	24.69 ± 3.92	55.67 ± 2.43	29.23 ± 2.34	15.49 ± 1.70
Heating (80°C, 30 min)	29.72 ± 2.86*	16.97 ± 4.64*	47.44 ± 2.62*	30.66 ± 3.87	13.48 ± 1.39
Heating (100°C, 15 min)	2.08 ± 0.55*	3.46 ± 1.15*	45.16 ± 2.86*	28.48 ± 2.41	8.30 ± 3.11*
Pepsin	5.55 ± 1.57*	2.80 ± 1.11*	32.39 ± 3.48*	34.51 ± 2.89	13.02 ± 1.06
Protease	16.78 ± 5.63*	17.16 ± 4.61*	55.62 ± 1.46	30.97 ± 5.51	15.36 ± 4.08
Trypsin	19.31 ± 3.25*	6.34 ± 2.03*	52.10 ± 3.14	32.57 ± 3.74	14.06 ± 3.81
Metaperiodate	38.19 ± 4.22	19.91 ± 2.77	48.60 ± 5.47	29.62 ± 6.32	8.41 ± 2.14*
Ca ²⁺	72.42 ± 7.19*	45.26 ± 2.90*	54.07 ± 5.63	33.06 ± 8.05	28.22 ± 4.78*
Mg ²⁺	41.15 ± 8.24	20.79 ± 5.37	50.37 ± 6.78	30.55 ± 7.12	15.63 ± 5.06

Results represent means of three independent experiments ± standard deviation.

*Significantly different from the control ($p < 0.05$).

capacity of *L. acidophilus* GK20 and *L. paracasei* GK74 was almost completely inhibited by heat treatment for 15 min at 100°C, the cell structures involved in the adhesion of the strains must be sensitive to heat. On the other hand, the adhesion ability of *L. brevis* MLK27 did not change by heating for 15 min at 100°C. Tuomola *et al.* [33] reported that the adhesion of *L. acidophilus* 1 and *L. rhamnosus* GG was reduced by boiling at 100°C and autoclaving at 125°C, but that of *L. casei* strain Shirota was not altered by heating treatment.

When the cells of *L. acidophilus* GK20, *L. paracasei* GK74, and *L. plantarum* GK81 were treated with pepsin, the adhesion of these strains was dramatically reduced. In addition, enzymatic treatments by protease and trypsin significantly lowered ($p < 0.05$) the adhesion capability of the strains *L. acidophilus* GK20 and *L. paracasei* GK74. However, pretreatment with these proteolytic enzymes did not influence the adhesion of *L. brevis* MLK27 and *P. pentosaceus* MLK67. These results were consistent with the results of Tuomola *et al.* [33], who showed that the adhesion of *L. acidophilus* LA1 was reduced by pepsin and trypsin treatments, suggesting that the bacterial protein structures are essential for their adhesion. The adherence of *L. acidophilus* BG2FO4 was significantly reduced by treatment with trypsin and pepsin, but was relatively unaffected by treatment with chymotrypsin. Therefore, putative surface layer proteins were involved in the adherence of the strain [10].

Proteolytic enzymes did not influence the adhesion of *P. pentosaceus* MLK67 strain to Caco-2 cells, but metaperiodate treatment significantly ($p < 0.05$) decreased the adhesion of this strain, suggesting that carbohydrates may be partially involved in the adhesion. The adhesion of *L. acidophilus* GK20, *L. paracasei* GK74, *L. plantarum* GK81, and *L. brevis* MLK27 strains was not altered by metaperiodate treatments. Greene and Klaenhammer [10] indicated that carbohydrate moieties are involved to varying degrees in the adherence of the strains examined, because periodate oxidation of cell surface carbohydrates had no effect on *L. acidophilus* NCFM/N2 adherence, moderately decreased *L. acidophilus* BG2FO4 adherence, and significantly reduced *L. gasseri* ADH adherence.

Addition of Ca^{2+} led to a significant ($p < 0.05$) increase of the adhesion of *L. acidophilus* GK20, *L. paracasei* GK74, and *P. pentosaceus* MLK67 strains. The largest effect was observed for *L. acidophilus* GK20 and *L. paracasei* GK74, for which the adhesion was increased by 31.70% and 22.19%, respectively, as compared with the controls. However, the adhesion ability of *L. plantarum* GK81 and *L. brevis* MLK27 was not different from the control by the addition of calcium. Meanwhile, no significant change in the adhesion of all the strains tested was observed upon addition of Mg^{2+} . Calcium ions are known to increase the adhesion of probiotic strains to surfaces of intestinal cells

and are involved in non-specific interactions such as neutralization of the electrical double layer between cell and substratum surface [4]. These findings are in agreement with Larsen *et al.* [14], who revealed that added calcium ions significantly increased the adhesion of tested lactobacilli to IPEC-J2 cells; in contrast, no significant change in the adhesion of lactobacilli was observed in the presence of Mg ions. Moreover, all the tested *Lactobacillus* strains reduced the attachment of pathogenic *E. coli* O138 to IPEC-J2 by more than 2-fold in the presence of calcium ions.

In conclusion, an extracellular proteinaceous component may be involved in the adhesion of *L. acidophilus* GK20 and *L. paracasei* GK74, since their adhesion was almost completely abolished by acidic pH, heating, and proteolytic enzymes. However, *P. pentosaceus* MLK67 adherence to Caco-2 cells seems related to carbohydrate factors, because the adhesion of this strain was significantly decreased by metaperiodate treatment.

Inhibition of Pathogen Adhesion to Caco-2 Cells

As a potential probiotic microorganism, the antimicrobial activity against pathogenic bacteria is an important property [7]. The results on the adhesion inhibition of pathogenic *S. Typhimurium* ATCC 29631 by the LAB tested are shown in Fig. 5. In the competition experiment, *L. plantarum* GK81 strain significantly prevented the adhesion of *S. Typhimurium* ATCC 29631 by $31.98 \pm 3.17\%$ ($p < 0.05$). The adhesion of *S. Typhimurium* ATCC 29631 was prevented by $16.85 \pm 3.17\%$ when co-incubated with *L. paracasei* GK74, and there was no significant difference between *L. paracasei* GK74 and *L. brevis* MLK27 for competitive inhibition of the pathogen. The adhering-displacement value of *L. paracasei* GK74 to detach Caco-2 cells-adherent *S. Typhimurium* ATCC 29631 was the highest ($16.06 \pm 4.69\%$) among the tested strains. The

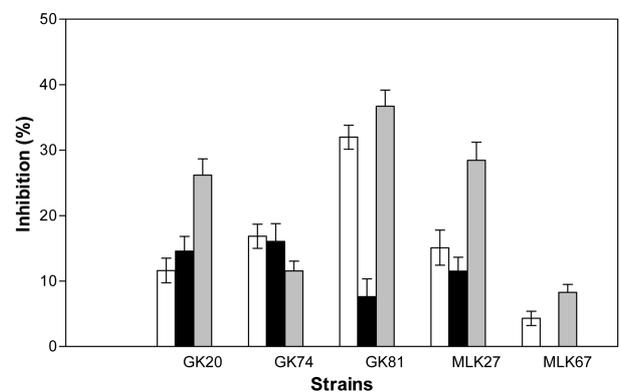


Fig. 5. Competition (□), displacement (■), and exclusion (■) inhibition of *S. Typhimurium* ATCC 29631 adhesion to Caco-2 cells by the LAB tested.

Results are expressed as means of three independent experiments and error bars represent standard deviations.

displaceable inhibition of the pathogen by *L. acidophilus* GK20 was similar to the inhibition ability by *L. paracasei* GK74. However, unlike the other strains, *P. pentosaceus* MLK67 did not exhibit the displacement profiles against the pathogen. Meanwhile, the strain *L. plantarum* GK81 showed significant levels ($p < 0.05$) of the exclusion activity, preventing $36.72 \pm 4.26\%$ of *S. Typhimurium* ATCC 133076 from adhering to Caco-2 cells. The exclusion inhibitions of *S. Typhimurium* by *L. acidophilus* GK20 and *L. brevis* MLK27 were $26.18 \pm 4.33\%$ and $28.46 \pm 4.78\%$, which were much higher than *L. paracasei* GK74 and *P. pentosaceus* MLK67. Although *L. paracasei* GK74 was the most effective strain displacing the pathogen, *L. plantarum* GK81 showed the highest inhibition of *S. Typhimurium* ATCC 29631 in both the competition and the exclusion experiments. These results indicated that the strains tested share the same binding sites for the attachment of the pathogen to Caco-2 cells. The adhesion of *L. plantarum* GK81 and *L. brevis* MLK27 to the mucosal surface occurs mainly *via* hydrophobic interactions; thus, the competition for a specific receptor that binds *S. Typhimurium* is due to steric hindrance.

The ability of probiotic LAB to displace the pathogens is important for prevention of the colonization of the intestine by harmful bacteria. Bernet *et al.* [3] demonstrated that the incubations of *L. acidophilus* LA1 before and together with enterovirulent *E. coli* were more effective than the incubation after infection by *E. coli*. Larsen *et al.* [14] considered that the adhesion inhibition of *E. coli* O157:H7 by the strains of *Lactobacillus* ssp. was most likely due to steric hindrance rather than to involvement of the specific binding sites.

The mechanism for the direct inhibitory activity of pathogenic bacteria might be the result of competition between probiotic and pathogen to nutritive substances or the induction of mucin production by probiotics. Chen *et al.* [5] suggested that the S-layer proteins from *L. crispatus* ZJ001 inhibited the adhesion of *S. Typhimurium* and *E. coli* O157:H7 to HeLa cells, and the proteins are involved in the adhesion and competitive exclusion of the pathogens to intestinal cell lines. Furthermore, anti-adhesiveness of the pathogens can be caused by secretion of antimicrobial substances (organic acids, bacteriocin, hydrogen peroxide, *etc.*) from the probiotic strains, degradation of carbohydrate receptors by secretion of proteins, establishment a biofilm, and production of receptor analogs and biosurfactants [22].

Interestingly, Gueimonde *et al.* [11] demonstrated that because the low-adhesive strain *L. rhamnosus* LA-2 to human intestinal mucus and Caco-2 cells was the most effective at inhibiting the adhesion of *L. monocytogenes* ATCC 15313, the inhibition was not related to the adhesion ability of the strains. Thus, the inhibition of the pathogens could be related with the specific receptors that probiotic and pathogen are competing for or with other factors such

as coaggregation of both strains. The displacement and the inhibition profiles of specific pathogens were also variable depending on both the lactobacilli and the pathogens. Sun *et al.* [32] suggested that cell surface-bound exopolysaccharides of *L. plantarum* Lp6 were involved in the adhesion capacity, and the mannose-specific adhesion of the strain might be important for competing with pathogens-binding sites in the intestinal tract and for resisting the pathogens colonization.

In summary, because *L. acidophilus* GK20, *L. paracasei* GK74, and *L. plantarum* GK81 had high tolerance to low pH and bile-salt conditions, and adhesion capacity to Caco-2 cells, these strains might have the ability to establish themselves in the gastrointestinal tract environment by surviving in the low pH of the stomach and colonizing the small intestine and colon. The mechanism and factors associated with attachment of these strains adhering to Caco-2 cells vary depending on the type of strains. In particular, the positive effect of Ca^{2+} on the adhesion of *L. acidophilus* GK20 and *L. paracasei* GK74 might be useful for development of probiotic foods. In other words, if these strains are used together with calcium-rich ingredients or calcium-supplemented foods, the adhesion of the strains to intestinal epithelial cells may be improved. These strains were also found to be effective in reducing adhesion of *S. Typhimurium* ATCC 29631 to Caco-2 cells, indicating their possible protective role against infection of the pathogen. Accordingly, *L. acidophilus* GK20 and *L. plantarum* GK81 have the potential as probiotics to improve the health status of hosts, although more information about the host/microbe interactions *in vivo* is still needed.

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