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ORIGINAL ARTICLE

Assessment of cell surface properties and adhesion potential of selected probiotic strains

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Abstract

Aim: To evaluate the physicochemical cell surface and adhesive properties of selected probiotic strains for human use.
Methods and Results: Probiotic strains, *Bifidobacterium longum* B6, *Lactobacillus acidophilus* ADH, *Lactobacillus paracasei*, *Lactobacillus rhamnosus* GG, *Lactobacillus brevis*, *Lactobacillus casei*, *Leuconostoc mesenteroides* and *Pediococcus acidilactici* were tested for the physicochemical properties of cell surfaces and the adhesion abilities against foodborne pathogens. *Bif. longum* B6 (53·6%) and *Lact. rhamnosus* GG (46·5%) showed the highest hydrophobicity, while the least affinity to xylene was observed in *Ped. acidilactici* (10·4%). *Bifidobacterium longum* B6 showed the strongest coaggregation phenotype with *Listeria monocytogenes* (53·0%), *Shigella boydii* (42·0%) and *Staphylococcus aureus* (45·9%). *Lactobacillus rhamnosus* GG had the strong binding ability to Caco-2 cells and effectively inhibited the adhesion of *L. monocytogenes*, *Salmonella* Typhimurium, *Sh. boydii* and *Staph. aureus* to Caco-2 cells. The hydrophobicity was highly correlated with coaggregative abilities and competitive inhibition, suggesting a good relationship between *in vitro* adhesion and *in vivo* colonization.
Conclusion: The results suggest that *Bif. longum* B6 and *Lact. rhamnosus* GG can be candidate probiotics available for human consumption.
Significance and Impact of the Study: Because the use of probiotic strains has been more concerned with their beneficial effects in the GI tract, it is essential to examine the potential of probiotic strains based on the physicochemical properties in terms of bacterial-binding and adhesion capabilities.

Introduction

The mucosal surfaces of the gastrointestinal tract (GIT) are particularly susceptible to adherence and colonization of foodborne pathogens. Foodborne infectious diseases caused by *Salmonella* Typhymurium, *Listeria monocytogenes*, *Staphylococcus aureus* and *Shigella* spp. include salmonellosis, listeriosis, staphylococcal infection and shigellosis. They can lead to several clinical syndromes such as gastroenteritis, mucosal ulceration, rectal bleeding, septicemia, endocarditis, osteomyelitis, nosocomial bacteremias, pneumonia, meningitis, arthritis and abscesses (Goosney *et al.* 1999; Ryser and Marth 1999; Le Loir *et al.* 2003; Foley and Lynne 2008). Pathogen infections are initiated by adhesion and further involved in invasion through multiple mechanisms that require filamentous structures, cell surface proteins, site-specific ligands or biofilms (Mengaud *et al.* 1996; Donlan and Costerton 2002; Seveau *et al.* 2007; Egea *et al.* 2008). Because adhesion is necessary for the initiation of host–pathogen interactions, preventing pathogenic bacterial adhesion to the epithelial cells of GIT is an effective strategy for reducing the risk of foodborne illness.

Probiotics, 'a live feed supplements which beneficially affect the host by improving its intestinal microbial balance' (Fuller 1989; Collado *et al.* 2007a), are attracting much attention in the food industry. Probiotic strains such as *Lactobacilli* and *Bifidobacteria* are autochthonous inhabitants of the GIT of humans (Collins and Gibson 1999; Corr *et al.* 2007). Although the exact mechanisms by which

probiotics provide various health benefits in the GIT have not been clearly understood, the major functions of probiotics include production of antimicrobials, stimulation of mucosal immunity, modulation of intestinal microflora and competitive exclusion of pathogens (Ketley 1997; Collano *et al.* 2006; Ng *et al.* 2009). Therefore, the use of probiotic strains is realistically considered as an alternative for improving intestinal microbial balance because of their nonpathogenic, safe and health beneficial properties (Fuller 1989; Collado *et al.* 2007a). However, to exert the beneficial effects for human health, the number of probiotics that reach the GIT is recommended to be more than 6 log CFU g^{-1} (Shah 2000). Because of the adverse environmental conditions, probiotic strains are less likely to colonize the GIT (Guglielmetti *et al.* 2008).

The ability to adhere to mucus and epithelial cells is proposed as an important selection criterion for potential probiotic strains. The adhesion ability of probiotic strains has been studied *in vitro* model systems, which are commonly used to select and assess probiotic strains for *in vivo* studies (Dunne *et al.* 2001; Collado *et al.* 2007c). The physicochemical properties of probiotic strains, however, do not always correspond to the adhesion to mucus and epithelial cells (Schillinger *et al.* 2005; Alzate *et al.* 2008). The adhesion of probiotic strains varies among strains, depending on the cell surface properties such as hydrophobicity and extracellular protein profiles (Botes *et al.* 2008). Therefore, the objective of this study was to evaluate the physicochemical properties and adhesion ability of probiotic strains available for human use, as measured by hydrophobicity, auto-aggregation, coaggregation and adhesion assays. The previously selected probiotic strains, *Bifidobacterium longum* B6, *Lactobacillus acidophilus* ADH, *Lactobacillus paracasei* ATCC 25598 and *Lactobacillus rhamnosus* GG, used in this study have known to exert antagonistic activities *in vitro* (Lee *et al.* 2008).

Materials and methods

Bacterial strains and culture conditions

Strains of *Bifidobacterium longum* B6, *Lactobacillus acidophilus* ADH, *Lactobacillus paracasei* ATCC 25598 and *Lact. rhamnosus* GG were kindly provided by Dr Azlin Mustapha of the Department of Food Science at the University of Missouri-Columbia. *Lactobacillus brevis* (KACC 10553), *Lactobacillus casei* (KACC 12413), *Leuconostoc mesenteroides* (KACC 12312) and *Pediococcus acidilactici* (KACC 12307) were obtained from the Korean Agricultural Culture Collection (KACC; Suwon, Korea). Probiotic strains were anaerobically grown in de Man, Rogosa, Sharpe (MRS; Difco, BD Diagnostic Systems, Sparks, MD, USA) broth supplemented with 0·05% cysteine-HCl at 30°C or 37°C for 24 h, conducted in a GasPak anaerobic system (BBL, Cockeysville, MD, USA) with AnaeroGen (Oxoid Ltd., Hampshire, UK). Pure cultures were harvested by centrifugation at 3000 ***g*** for 20 min at 4°C. The pellets were used for hydrophobicity, aggregation and adhesion assays. Pathogenic strains of *L. monocytogenes* (KACC 12671), *Staph. aureus* (KACC 10768), *Shigella boydii* (KACC 10792) and *Salmonella* Typhimurium (KCCM 40253) were provided by the Korean Agricultural Culture Collection (KACC) and Korean Culture Center of Microorganisms (KCCM; Seoul, Korea). The strains were cultivated in trypticase soy broth supplemented with 0·1% yeast extract (TSBYE; BD, Becton, Dickinson and Co., Sparks, MD, USA) at 37°C for 20 h. After cultivation, cultures were harvested at 3000 ***g*** for 20 min at 4°C.

Bacterial adhesion to solvents

The bacterial adhesion to solvent (BATS) assay with slight modification was used to determine cell surface properties (Bellon-Fontaine *et al.* 1996; Kos *et al.* 2003). The adhesion to xylene (apolar solvent) demonstrates the hydrophobic surface characteristic of bacteria. The affinities to chloroform (polar acidic solvent) and ethyl acetate (polar basic solvent) describe the electron donor and electron acceptor properties of the bacterial cell surface, respectively. Bacterial cells were suspended in phosphate-buffered saline (PBS; pH 7·2) to *c.* 10^8 CFU ml^{-1}. The cell suspension (3 ml) was mixed with 1 ml of solvent; xylene as an apolar solvent, chloroform as an electron acceptor, or ethylene acetate as an electron donor. The mixture was vortexed for 1 min and allowed to stand for 5 min to separate into two phases. The aqueous phase was measured at 600 nm using Labomed UV 2800 spectrophotometer (Labomed Inc., Culver City, CA, USA). The affinities to solvents with different physicochemical properties (hydrophobicity and electron donor–electron acceptor interactions) were expressed using the

$$\mathrm{BATS}\,(\%) = (1 - A_{5\,\mathrm{min}}/A_{0\,\mathrm{min}}) \times 100.$$

Auto-aggregation and coaggregation assays

The specific cell–cell interactions were determined using auto-aggregation assay (Del Re *et al.* 2000) and coaggregation assay (Handley *et al.* 1987). The bacterial cells were harvested at 5000 ***g*** for 10 min at room temperature, washed with PBS and resuspended in PBS to *c.* 10^8 CFU ml^{-1}. For the auto-aggregation assay, each

bacterial suspension (3 ml) was vortexed for 10 s and incubated at 37°C for 2 h. The absorbance of the supernatant was measured at 600 nm using Labomed UV 2800 spectrophotometer. The auto-aggregation was calculated with the following:

$$\text{Auto-aggregation}\,(\%) = (1 - A_{2\,\mathrm{h}}/A_{0\,\mathrm{h}}) \times 100.$$

For the coaggregation assay, equal volumes (1·5 ml) of probiotic strain and pathogenic bacterium were mixed, vortexed for 10 s and incubated at 37°C for 2 h. The supernatants were measured at 600 nm. The coaggregation was calculated according to the following:

$$\text{Coaggregation}\,(\%) = [1 - A_{\mathrm{mix}}/(A_{\mathrm{probiotic}} + A_{\mathrm{pathogen}})/2] \times 100.$$

Caco-2 cell culture

Caco-2 cell line (KCLB 30037), originated from a human colonic adenocarcinoma, was purchased from the Korean Cell Line Bank (KCLB, Seoul, Korea). The cells were cultured in Dulbecco's modified Eagle's minimal essential medium (DMEM; HyClone Laboratories Inc., Logan, UT, USA) supplemented with 10% heat-inactivated foetal bovine serum (FBS; HyClone), L-glutamine (2 mmol l^{-1}), penicillin (100 U ml^{-1}) and streptomycin (100 μg ml^{-1}) in the incubator with 95% (v/v) humidified air and 5% (v/v) CO_2 at 37°C. For adhesion assays, the Caco-2 cells were seeded at 2×10^5 cells ml^{-1} (preconfluence) in 24-well tissue culture plates and fully differentiated for 16 days (postconfluence) by changing the culture medium every 2–3 days. The cells maintained in the confluent state were replaced by fresh nonsupplemented DMEM for 1 h prior to the adhesion assay and then rinsed three times with the DMEM medium.

Adhesion assay

For the adhesion assessment, each probiotic strain (or foodborne pathogen) was inoculated at *c.* 10^8 CFU ml^{-1} into Caco-2 cells grown in late postconfluence. The inoculated 24-well plates were incubated for 2 h at 37°C. After incubation, the nonadherent probiotics and pathogens were removed by washing two times with 1% sterile peptone water. The Caco-2 cell monolayers were treated with 0·05% trypsin-EDTA. To enumerate the adherent probiotics and pathogens, the cell lysates were serially (1 : 10) diluted with 0·1% peptone water. The serial dilutions of probiotic strains, *L. monocytogenes*, *Staph. aureus*, *Sh. boydii* and *Salm.* Typhimurium were pour-plated on MRS agar, modified oxford agar, baird-parker agar and xylose lysine deoxycholate agar, respectively, and incubated at 37°C for 48 h. Percent inhibition was estimated by the number of probiotics (or pathogens) adhered to Caco-2 cells when compared to the inoculum level.

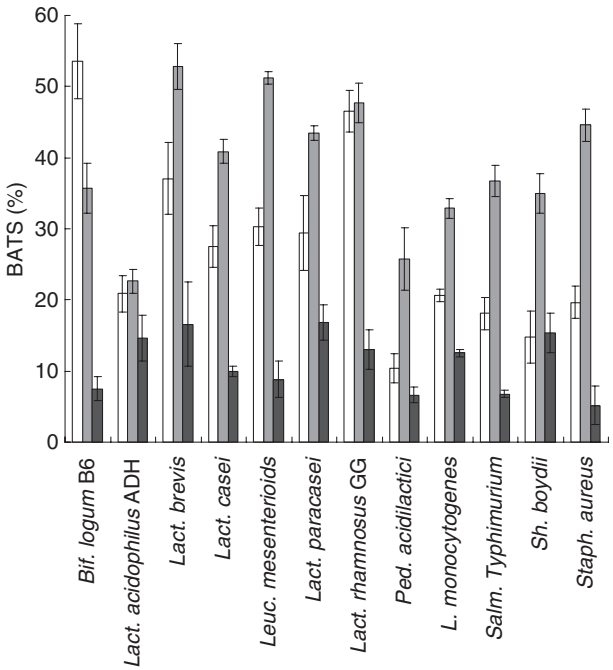

Figure 1 Hydrophobicity (□), electron donor (▩) and electron acceptor (■) characteristics of probiotic strains and foodborne pathogens (different letters are significantly different at $P < 0{\cdot}05$).

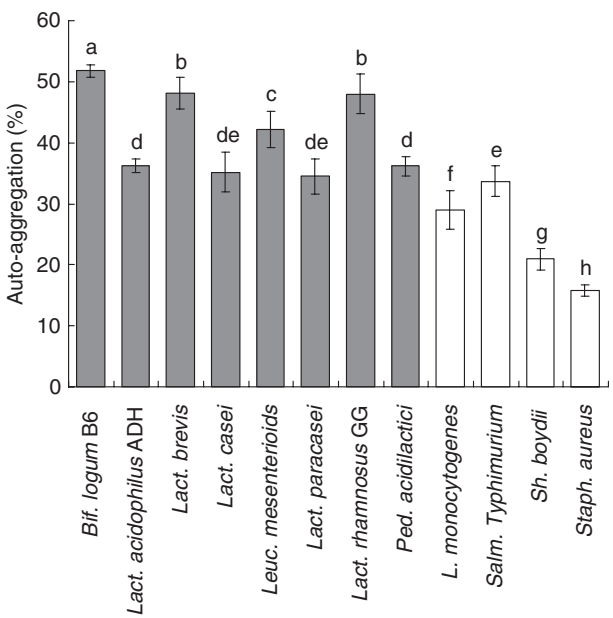

Figure 2 Autoaggregation abilities of probiotic strains (▩) with different foodborne pathogens (□) after 2-h incubation at 37°C.

Competitive inhibition assay

To evaluate the competitive ability, probiotic and pathogen (*c.* 10^8 CFU ml^{-1} each) were cocultured in each well of Caco-2 cell monolayers for 2 h. After cultivation, the suspensions were discarded and washed two times with 0·1% peptone water. The Caco-2 cell monolayers were treated with 0·05% trypsin-EDTA. The pathogens adhered to Caco-2 cells were serially (1 : 10) diluted with 0·1% peptone water and then spread-plated on each selective agar. The adhesion inhibition was calculated by the number of pathogens adhered to Caco-2 cells compared to the number of pathogens adhered in the absence of probiotic strains.

Statistical analysis

Each experiment was conducted with three replicates. Data were analysed using the Statistical Analysis System software. The general linear model and least significant difference (LSD) procedures were used to evaluate the treatment as a fixed effect. Significant mean differences were calculated by Fisher's LSD at $P < 0{\cdot}05$. Correlation coefficients were calculated between physicochemical properties and adhesion ability *in vitro*, and statistical difference was established at $P < 0{\cdot}05$, $P < 0{\cdot}01$ and $P < 0{\cdot}001$.

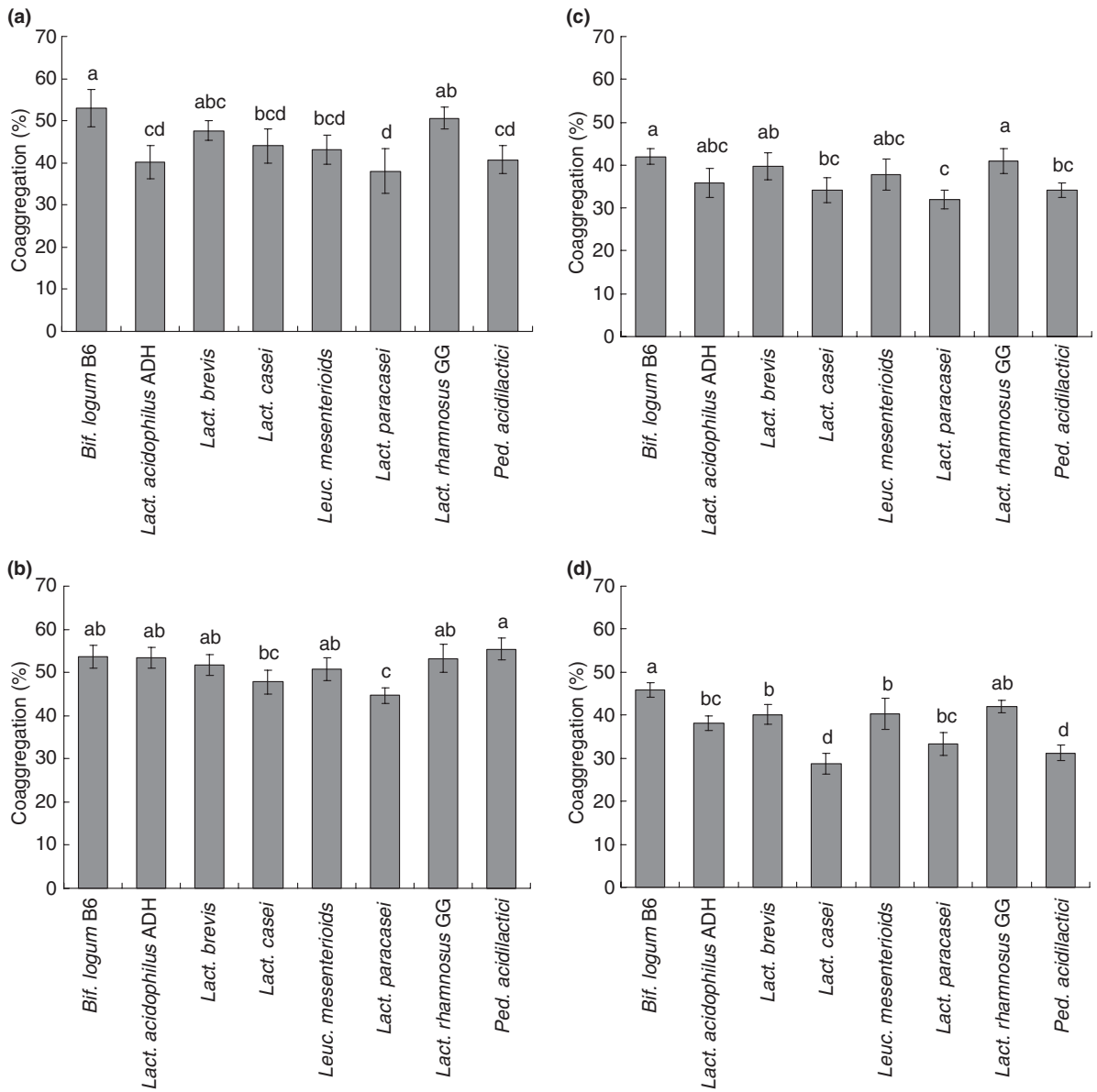

Figure 3 Coaggregation abilities of probiotic strains and foodborne pathogens, *Listeria monocytogenes* (a), *Salmonella* Typhimurium (b), *Shigella boydii* (c) and *Staphylococcus aureus* (d) after 2-h incubation at 37°C.

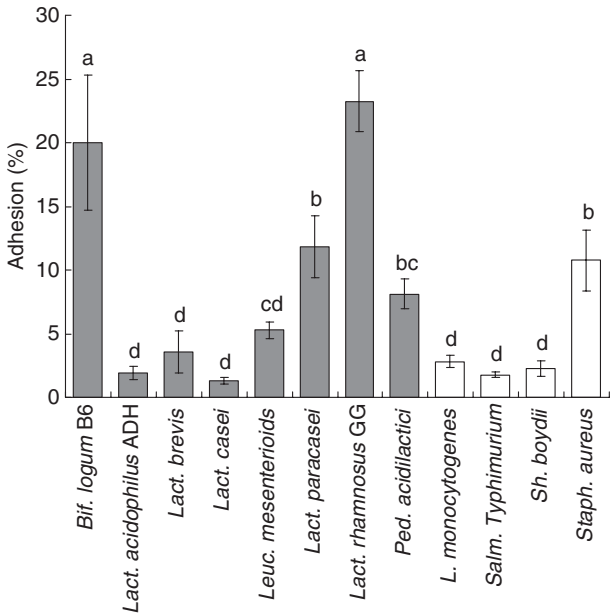

Figure 4 Adhesion of probiotic strains (■) and foodborne pathogens (□) to Caco-2 cells after 2-h incubation at 37°C.

Results

Physicochemical properties of bacterial cell surface

The adhesive characteristics of probiotic strains and foodborne pathogens to xylene, chloroform and ethyl acetate are shown in Fig. 1. Most strains of probiotics, except for *Ped. acidilactici* (10·4%), showed relatively higher hydrophobicity than foodborne pathogens. The most hydrophobic strains were *Bif. longum* B6 (53·6%) and *Lact. rhamnosus* GG (46·5%), followed by *Lact. brevis* (37·1%), *Leu. mesenteroides* (30·3%) and *Lact. casei* (27·5%). When compared to the hydrophobicity, the affinities with chloroform were significantly increased in both probiotic strains and pathogens, while those with ethyl acetate were decreased ($P < 0·05$). The greatest affinities with chloroform were observed in *Lact. brevis* (52·9%), *Leu. mesenteroides* (51·2%) and *Lact. rhamnosus* GG (47·7%), while the least affinities were observed in *Lact. acidophilus* ADH (22·6%) and *Ped. acidilactici* (25·8%). Unlike chloroform, the bacterial adhesion to ethyl acetate was low, ranging from 5·1 to 16·9%.

Bacterial auto-aggregation and coaggregation abilities

The auto-aggregation properties of probiotic strains and foodborne pathogens are shown in Fig. 2. The probiotic strains showed higher auto-aggregation values, ranging between 36·2 and 51·8%, than the food foodborne pathogens at 37°C. *Bifidobacterium longum* B6 showed the greatest auto-aggregative ability (51·8%, $P < 0·05$), whereas *Staph. aureus* showed the least auto-aggregative ability (15·8%). The most auto-aggregative strain was *Salm*. Typhimurium (33·7%) among pathogens. The coaggregation between probiotic strains and foodborne pathogens is shown in Fig. 3. Among the probiotic strains tested, *Bif. longum* B6 showed the highest coaggregation abilities with *L. monocytogenes* (53·1%), *Sh. boydii* (42·0%) and *Staph. aureus* (45·9%) with the exception of *Ped. acidilactici*. *Pediococcus acidilactici* showed the most coaggregation ability with *Salm*. Typhimurium (55·4%). All probiotic strains tested were highly coaggregated with *Salm*. Typhimurium (44·7–55·4%). Among the probiotic strains, *Lact. paracasei* showed the least coaggregation abilities with *L. monocytogenes* (38·0%), *Salm*. Typhimurium (44·7%) and *Sh. boydii* (32·0%). *Lactobacillus casei* demonstrated the least coaggregation ability with *Staph. aureus* (28·7%). In general, the hydrophobicity was related to the auto-aggregation and coaggregation.

Adhesion of and competition between probiotic strains and pathogens to Caco-2 cells

The adhesion abilities of probiotic strains and foodborne pathogens to Caco-2 cells are shown in Fig. 4. The probiotic strains and pathogens were inoculated at *c.* 10^8 CFU ml^{-1}. The most adhesive strains were *Lact. rhamnosus* GG (23·2%) and *Bif. longum* B6 (20·0%), followed by *Lact. paracasei* (11·9%), *Staph. aureus* (10·8%) and *Ped. acidilactici* (8·1%). Among the probiotic strains *Lact. acidophilus* ADH, *Lact. brevis* and *Lact. casei* were less adhered to Caco-2 cells (<6·0%). The adhesion rates of *L. monocytogenes*, *Salm*. Typhimurium and *Sh. boydii* were 2·8%, 1·7% and 2·3%, respectively. The competitive inhibition of adhesion of foodborne pathogens to Caco-2 cells by probiotic strains was shown in Fig. 5. The probiotic strains tested competitively inhibited the adhesion of *L. monocytogenes* (13·8–43·6%), *Salm*. Typhimurium (3·2–25·9%), *Sh. boydii* (3·6–23·8%) and *Staph. aureus* (4·2–40·1%). The adhesion of *L. monocytogenes*, *Salm*. Typhimurium, *Sh. boydii* and *Staph. aureus* to Caco-2 cells was significantly inhibited by *Lact. rhamnosus* GG. The competitive inhibition of *L. monocytogenes* was not observed in the presence of *Lact. acidophilus* ADH, *Lact. brevis* and *Lact. casei*, showing negative inhibition rates (Fig. 5).

Discussion

This study demonstrates the cell surface characteristics, binding properties and adhesion abilities of selected probiotic strains and foodborne pathogens.

Xylene, chloroform and ethyl acetate were used to assess the hydrophobic/hydrophilic, electron donor

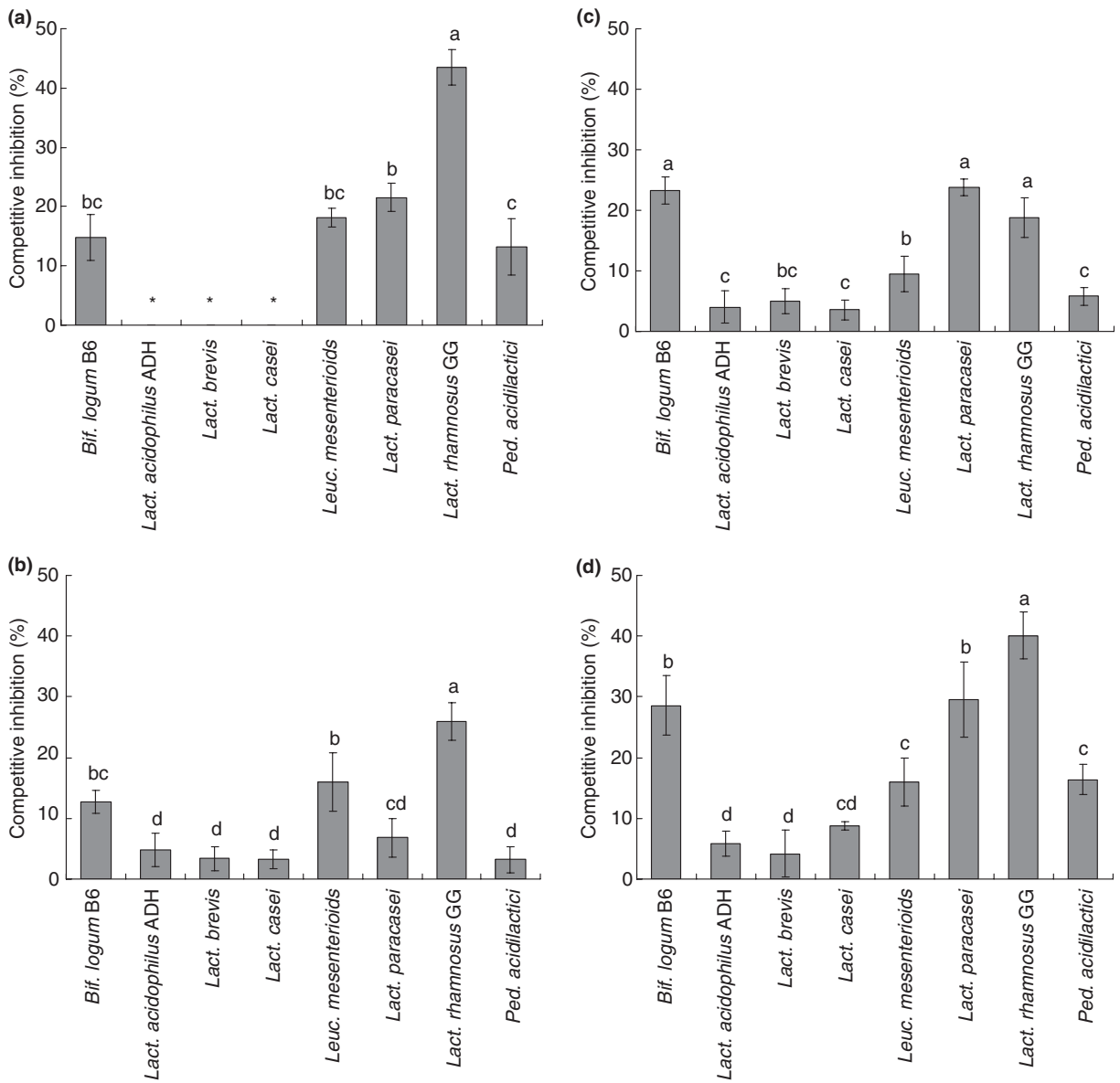

Figure 5 Competitive inhibition of adhesion of *Listeria monocytogenes* (a), *Salmonella* Typhimurium (b), *Shigella boydii* (c) and *Staphylococcus aureus* (d) to the Caco-2 cells by probiotic strains (*indicates negative value).

(basic) and electron acceptor (acidic) characteristics of bacterial surface, respectively (Fig. 1), which are attributed to carboxylic groups and Lewis acid–base interactions (Bellon-Fontaine *et al.* 1996; Kos *et al.* 2003). *Bifidobacterium longum* B6 and *Lact. rhamnosus* GG showed higher hydrophobicity, while *L. monocytogenes* and *Salm.* Typhimurium showed lower hydrophobicity. The hydrophobic differences between probiotics and pathogens may result in colonizing ability. The hydrophobic and hydrophilic properties are resulted from proteins and polysaccharides on the bacterial cell surface (Chauviere *et al.* 1992). The bacterial affinities to ethyl acetate were relatively low when compared to xylene and chloroform, indicating probiotic strains and foodborne pathogens have the nonacidic and poor electron acceptor property (Pelletier *et al.* 1997). Most probiotic strains tested showed relatively higher auto-aggregation than the pathogens (Fig. 2), suggesting specific binding capabilities of probiotics in the GIT. *Bifidobacterium longum* B6 showed strong auto-aggregation ability and was also well coaggregated with the foodborne pathogens tested. This result suggests that the coaggregation property is related

Table 1 Correlation matrix of Pearson coefficients between cell surface properties and *in vitro* adhesion (n = 16)

Assay	Bacterial adhesion to solvent			Auto-aggregation	Adhesion
	Xylene	Chloroform	Ethyl acetate		
Coaggregation					
Listeria monocytogenes	0·739**	0·297	0·102	0·825***	0·569*
Salmonella Typhimurium	0·030	0·393	0·273	0·487	0·245
Shigella boydii	0·630*	0·247	0·019	0·933***	0·502*
Staphylococcus aureus	0·701**	0·219	0·091	0·877***	0·545*
Competitive inhibition					
L. monocytogenes	0·409	0·376	0·142	0·309	0·799***
Salm. Typhimurium	0·558*	0·388	0·045	0·564*	0·720**
Sh. boydii	0·601*	0·199	0·058	0·347	0·779***
Staph. aureus	0·501*	0·166	0·041	0·348	0·921***

*Level of significance at $P < 0·05$.
**Level of significance at $P < 0·01$.
***Level of significance at $P < 0·001$.

to the auto-aggregation ability of each strain (Collado *et al.* 2007b). The coaggregation ability can help prevent colonization by invading foodborne pathogens.

The enterocyte-like Caco-2 cell model is commonly used to investigate the adhesion inhibition, displacement and competitive inhibition because the adhesion ability to epithelial cells is primarily considered a functional criterion for the selection of potential probiotic strains (Greene and Klaenhammer 1994). *Bifidobacterium longum* B6 and *Lact. rhamnosus* GG strongly adhered to Caco-2 cells (Fig. 4) and effectively inhibited the adherence of pathogens to Caco-2 cells (Fig. 5). The observation suggests that *in vitro* adhesion to Caco-2 cells is correlated with competitive inhibition, which is competitively excluding foodborne pathogens. Bacterial adhesion to the GIT is a complex mechanism that involves extracellular and cell surface receptors (Greene and Klaenhammer 1994; Kos *et al.* 2003). The adhesion of invading pathogens can also be affected by probiotic metabolites such as organic acids, hydrogen peroxide, peptides, polysaccharides and bacteriocins (Bogovic-Matijasic *et al.* 1998; Lee *et al.* 2000). The reduction in pathogen adhesion by *Bif. longum* B6, *Leu. mesenteroides*, *Lact. paracasei*, *Lact. rhamnosus* GG and *Ped. acidilactici* may be because of the steric hindrance that probiotic strains compete with pathogens for attachment sites (adhesion–receptor interactions) (Lee and Puong 2002).

The correlation coefficients between bacterial cell surface characteristics (BATS), cell-binding properties (auto-aggregation and coaggregation) and adhesion abilities (*in vitro* adhesion to Caco-2 cells) were shown in Table 1. The auto-aggregation was highly correlated with the coaggregation of probiotics with all pathogens, except for *Salm.* Typhimurium ($R^2 = 0·487$, $P > 0·05$). The adhesion to xylene (hydrophobicity) well correlated with the coaggregation and the competitive inhibition, respectively, except for *Salm.* Typhimurium and *L. monocytogenes*. The results confirm previous study suggesting that the competitive inhibition of *Salm.* Typhimurium was attributed to steric hiderance (Chauviere *et al.* 1992). *In vitro* adhesion to Caco-2 cells was highly correlated with the competitive inhibition. The affinity to xylene was highly correlated with the auto-aggregation ($R^2 = 0·771$, $P < 0·001$) and the ability of adhesion to Caco-2 cells ($R^2 = 0·662$, $P < 0·01$) (data not shown), indicating that the hydrophobicity of bacterial surface can be a good indicator for screening potential probiotic strains. The adhesion ability was correlated to the auto-aggregative ability ($R^2 = 0·581$, $P < 0·05$) (data not shown). The general correlation between *in vitro* adhesion and *in vivo* colonization has been proposed (Guglielmetti *et al.* 2008). Therefore, the most significant finding in this study was that *Bif. longum* B6 and *Lact. rhamnosus* GG can be used as potential probiotics to promote the GI colonization. Once a stable and balanced microflora is established, the gut flora and their metabolites may contribute to the homeostasis and colonization associated with the competitive exclusion in the GIT. This study would provide useful information for screening possible probiotic strains, based on the correlation between physicochemical properties, bacterial-binding abilities and adhesion to Caco-2 cells. Therefore, further studies are needed to understand phenotype variations in strains, species and genera in terms of cell surface properties, adhesion and competitive exclusion for *in vivo* selection criteria.

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