Journal of Microbiology (2013) Vol. 51, No. 5, pp. 584–589
DOI 10.1007/s12275-013-2632-9

Live/Dead State Is Not the Factor Influencing Adhesion Ability of *Bifidobacterium animalis* KLDS2.0603

Li-Qun Wang[1,2], Feng Zhao[1], Fei Liu[1], and Xiang-Chen Meng[1,2]*

[1]*Key Laboratory of Dairy Science (Ministry of Education), Northeast Agricultural University, Harbin 150030, P. R. China*
[2]*Food Science and Technology College, Northeast Agricultural University, Harbin 150030, P. R. China*

(Received November 16, 2012 / Accepted April 22, 2013)

Two essential requirements for probiotic bifidobacteria are that they be "live" and have "colonization" ability, following FAO/WHO guideline recommendations. The amount of research on the adhesion ability of bifidobacteria compares poorly with that of other probiotic bacteria, such as lactobacilli. The aim of the present study was to determine how gastrointestinal conditions affect the adhesion ability of bifidobacteria, and to investigate the relationship between the adhesion ability and the live/dead state of bifidobacteria. The adhesion ability of *Bifidobacterium animalis* KLDS2.0603 that had been subjected to the digestive enzymes, pepsin, trypsin, and proteinase K, was decreased significantly, but these treatments did not significantly change the strain's survival rates, which were 98.78%, 97.60%, and 97.63% respectively. *B. animalis* KLDS2.0603 subjected to LiCl retained its adhesion ability but had a lower survival rate (59.28%) than the control group ($P<0.01$). *B. animalis* KLDS 2.0603 subjected to sodium metaperiodate exhibited higher adhesion ability than the control group ($P<0.01$), but the bacterial cells were killed totally. The results of transmission electron microscopy and laser scanning confocal microscopy showed that live/dead state of bifidobacteria was not one of the main factors that affected the adhesion ability of bifidobacteira, and that the substances affecting the adhesion ability of bifidobacteria were on the outer surface layer of the bifidobacterial cells. Our results also indicated that the substances related to the adhesion ability of bifidobacteria are proteinaceous. The above results will help us to understand the adhesion and colonization processes of bifidobacteria in the human gastrointestinal tract.

Keywords: *Bifidobacterium animalis*, adhesion, colonization, live/dead

*For correspondence. E-mail: xchmeng@hotmail.com; Tel.: +86-451-5519-1813; Fax: +86-451-5519-0340

Introduction

The adhesion and colonization abilities of bifidobacteria are known to be critical prerequisites for performing their physiological functions, such as, pathogenic microorganism exclusion and host immune modulation by initiating cell signaling with the immune system (Candela *et al*., 2005; Frece *et al*., 2005a; Collado *et al*., 2008; Jankowska *et al*., 2008). Therefore, adhesion to the intestinal mucosa is one of the essential requirements for screening probiotic bifidobacterial strains. According to a FAO/WHO (2001) report, probiotics can be defined as "living microorganisms, which exert health benefits beyond inherent general nutrition". It is universally known that probiotics are always subjected to adverse conditions when they pass through the gastrointestinal tract, including gastric acid, bile salts and digestive enzymes. The bacterial survival rates have been estimated at 20–40% for certain strains (Bezkorovainy, 2001). A remaining question is whether the adhesion ability of dead bifidobacterial cells differs from that of live cells? The relationship between adhesion ability and the live/dead state of bifidobacterial cells has not been extensively studied.

Several methods are currently used for the determination of bacterial adhesion ability, the most common methods being Gram staining and plate counting. Compared to Gram staining, plate counting is time-consuming and only enumerates viable cells (Le Blay *et al*., 2004). Therefore, Gram staining is always used to determine the adhesion ability of the total live and dead bacterial cells. It is known that cell surface structures are important factors in adhesion and colonization (Deepika *et al*., 2009; Watanabe *et al*., 2010; Samot *et al*., 2011). Nevertheless, there is little direct evidence regarding whether the components related to the adhesion ability of bifidobacteria are on the cell surface (Collado *et al*., 2007). To identify factors potentially involved in bifidobacterial adhesion, such as (glyco-) protein and polysaccharides (Fontaine *et al*., 1994), we used selected enzymes and chemicals to treat the surface of the bifidobacterial cells. These treatments simulated the conditions that the bacteria experience in the gastrointestinal tract.

To date, studies on the mechanism underlying the adhesion ability of bifidobacteria have all been based on live bacteria (Ouwehand *et al*., 2001; Collado *et al*., 2007). To further understand the mechanism by which bifidobacteria adhere to human intestinal cells, we investigated the adhesion ability, and the survival rate, of *B. animalis* KLDS2.0603 subjected to different enzymatic and chemical treatments *in vitro*. Laser scanning confocal microscope (LSCM), combined with a novel and substantially modified application of a double-staining procedure, was used to examine the ad-

hesion ability of dead bifidobacterial cells, which has never before been successfully applied to study the adhesion ability of bifidobacteria. To gain information on the structural characteristics of the cell surface, and understand the relationship between adhesion ability and the live/dead state of bifidobacterial cells, we investigated the variation of surface layers of *B. animalis* KLDS2.0603 subjected to various enzymes and chemicals, using transmission electron microscopy (TEM) and LSCM.

Materials and Methods

Bacterial growth conditions and cell culture

B. animalis KLDS2.0603 was grown in modified MRS (mMRS) broth (Oxoid Ltd., England) supplemented with 0.05% L-cysteine hydrochloride, and was incubated at 37°C for 24 h under anaerobic conditions (80% N_2, 10% CO_2, 10% H_2).

An epithelial intestinal cell line, Caco-2, was employed for the adhesion experiments (Candela *et al*., 2005). They were grown in Dulbecco's modified Eagle's Medium (DMEM) (Gibco™ Invitrogen, USA) supplemented with 10% heat-inactivated (56°C for 30 min) fetal bovine serum (Gibco™ Invitrogen) and 1% non-essential amino acids (Gibco™ Invitrogen), at 37°C in an atmosphere of 5% CO_2 and 95% air. The cell culture medium was changed twice weekly.

Analysis of the adhesion ability of *B. animalis* KLDS2.0603 to Caco-2 cells *in vitro*

Cells were seeded at a concentration of 1×10^5 cells/well in 6-well tissue culture plates (NUNC, Denmark). The cell culture medium (2 ml/well) was changed every 2 days and 24 h before an adhesion assay. Cells were used for adhesion assays at late post-confluence, i.e., after 15–17 days in culture and complete differentiation. The culture medium was replaced by an antibiotic-free medium, the day before the adhesion assay. Cell monolayers were carefully washed twice with phosphate-buffered saline (PBS: 140 mM NaCl, 3 mM KCl, 8 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.2) before bacterial cells were added (Le Blay *et al*., 2004).

Approximately 10^8 cells of *B. animalis* KLDS2.0603 were harvested by centrifugation (8,000×g, 10 min) and washed once in PBS (pH 7.2), then suspended in 1 ml of one of the following solutions: control buffer (PBS, pH 7.2); 0.1 M phosphate buffer (pH 2.8); 0.1 M acetate buffer (pH 4.5); 1 g/L trypsin; 0.5 g/L pepsin; 0.5 g/L proteinase K; 5 M LiCl and 10 g/L sodium metaperiodate. After incubation for 1 h at 37°C, the bacterial cells were centrifuged at 8,000×g for 10 min, washed twice in 1 ml PBS (pH 7.2), and resuspended in 1.0 ml DMEM (pH 7.2). Then, the cells of *B. animalis* KLDS2.0603 were incubated with a monolayer of fully differentiated Caco-2 cells. After 1 h at 37°C in anaerobic conditions (5% CO_2 and 95% air), all monolayers were washed five times with PBS (pH 7.2) to release unbound bacteria, fixed with methanol, Gram stained and counted, using 20 randomized microscopic fields per well (Del Re *et al*., 2000). Data were expressed as number of bacteria adhering to 100 cells. Each adhesion assay was conducted independently, in triplicate.

Determination of survival rate of *B. animalis* KLDS2.0603

As described for the assay measuring *B. animalis* KLDS2.0603 ability to adhere to Caco-2, bacterial cell suspensions was prepared and treated by various enzymes and chemicals. Then, the cell suspensions were serially diluted 10-fold and plated onto mMRS agar. The plates were incubated at 37°C for 36 h under anaerobic conditions before enumeration. Each survival rate assay was conducted independently, in triplicate. The survival rate was calculated as follows:

$$\text{Survival rate (\%)} = [(\text{CFU/ml}) \text{ treatment} / (\text{CFU/ml}) \text{ control}] \times 100$$

Observation of the adhesion interaction between Live/Dead bifidobacterial cells and Caco-2 cells by LSCM

The LIVE/DEAD® BacLight™ bacterial viability kit (Molecular Probes, USA) that consisted of two separate stock solutions, SYTO9® and PI (propidium iodide), was used to determine the proportion of live/active cells (fluorescent green) and dead/inactive cells (fluorescent red) (Palencia *et al*., 2008). Stock solutions were dissolved in dimethyl sulfoxide at high concentration: 1.67 mM SYTO9® and 1.67 mM PI following the manufacturer's instructions.

B. animalis KLDS2.0603 was grown at 37°C in 30 ml mMRS broth to late log phase. Cells were collected from 25 ml of bacterial culture by centrifugation (10,000×g, 10 min), and the pellets were resuspended in 2 ml of 0.85% NaCl buffer. One milliliter of this suspension was added to each of two 50 ml centrifuge tubes containing either 20 ml of 0.85% NaCl (for live bifidobacterial cells) or 20 ml of 70% isopropyl alcohol (for dead bifidobacterial cells), and both bacterial samples were incubated at 25°C for 1 h with mixing every 15 min, then centrifuged at 10,000×g for 10 min to collect bacterial pellets. The pellets were washed twice with 0.85% NaCl and mixed together in a 1:1 (live cells:dead cells) proportion. Then, the adhesion ability of the mixture of live/dead *B. animalis* KLDS2.0603 was assayed as for *B. animalis* KLDS2.0603 adhesion to Caco-2, as described above. Cells of *B. animalis* KLDS2.0603 adhering to Caco-2 cells were stained with 500 µl fluorescence stain solution (SYTO9:PI = 1:1) and the adhesion ability of live/dead bifidobacterial cells to Caco-2 cells was determined microscopically at excitation/emission maxima 480/500 nm for SYTO9 and 490/635 nm for PI.

TEM examination of the surface of bifidobacterial cells subjected to certain treatments

Cells of *B. animalis* KLDS2.0603 were subjected to 8 different treatments as described above, and then washed twice with PBS (pH 7.2). After centrifugation (10,000×g, 10 min), the cell pellets were resuspended in PBS (pH 7.2). The solution of treated bacterial cells was then spotted onto a vegetable parchment and adsorbed by the film with a copper screen. Cells of *B. animalis* KLDS2.0603 adhering to the film was stained with 3% phosphotungstic acid and examined microscopically (magnification, ×40,000). The cells of bacteria treated with PBS (pH 7.2) were taken as the control.

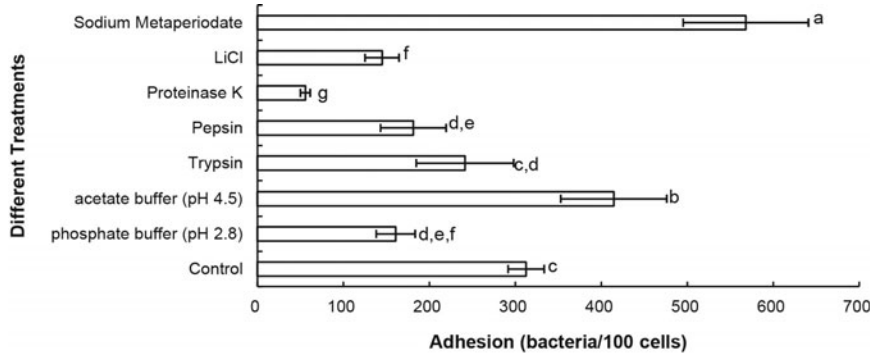

Fig. 1. Analysis of the adhesion ability of *B. animalis* KLDS2.0603 subjected to different treatments. The error bars represent standard deviations of the means (n=3). a, b, c, d, e, f, g indicates significantly different at $P<0.01$ level.

Examination of the adhesion of bifidobacteria subjected to different treatments

B. animalis KLDS2.0603 was grown for 24 h at 37°C in mMRS broth to reach a concentration of 10^8 CFU/ml, then centrifuged at 8,000×g for 10 min. The bacterial cell pellets were subjected to 8 different treatments as described above, resuspended in 2 ml of 0.85% NaCl buffer, and then the cells were incubated on a monolayer of a fully differentiated Caco-2 cell culture on the coverslips in 6-well tissue culture plates (Bernet *et al.*, 1993; Guglielmetti *et al.*, 2008). After 1 h at 37°C in anaerobic conditions, all monolayers were washed five times with 0.85% NaCl buffer to release unbound bacteria. Then the bacteria adhering to Caco-2 cells were stained with 500 µl fluorescence stain solution (SYTO9:PI = 1:1) and examined microscopically at 670 nm.

Statistical analysis

Means and standard deviations were calculated using the SAS 8.1 package (SAS Institute, USA). Significant differences between means were calculated at the $P=0.01$ level.

Results

Influence of different treatments on the adhesion ability of *B. animalis* KLDS2.0603

The adhesion ability of *B. animalis* KLDS2.0603 after 8 different treatments (PBS, phosphate buffer, acetate buffer, trypsin, pepsin, proteinase K, LiCl, sodium metaperiodate) was assessed after 1 h of incubation with Caco-2 cells and measured as the number of bacterial cells adhering to 100 Caco-2 cells. The results (Fig. 1) indicated that the adhesion abilities of bifidobacterial cells subjected to the phosphate buffer (pH 2.8), trypsin, pepsin, proteinase K, and LiCl treatments were lower than that for cells treated by the control buffer (PBS, pH 7.2) ($P<0.01$). In contrast, the adhesion abilities of the cells treated with acetate buffer (pH 4.5) and sodium metaperiodate were higher than cells treated with control buffer (PBS, pH 7.2) ($P<0.01$).

Determination of survival rate of *B. animalis* KLDS2.0603 after different treatments

Plate counting was used to evaluate the survival rates of *B. animalis* KLDS2.0603 subjected to different enzymatic and chemical treatments (Fig. 2). *B. animalis* KLDS2.0603 was sensitive to sodium metaperiodate, which killed all the bacterial cells (live bacteria were not detected). The survival rate of this strain decreased to 59.28% after LiCl treatment ($P<0.01$). The survival rates were reduced very little when bifi-

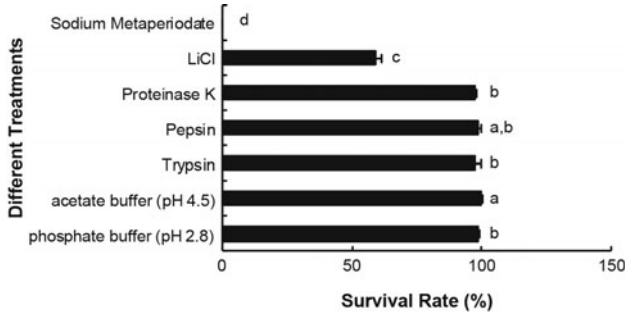

Fig. 2. Analysis of the survival rate of *B. animalis* KLDS2.0603 subjected to different treatments. The error bars represent standard deviations of the means (n=3). a, b, c, d indicates significantly different at $P<0.01$ level.

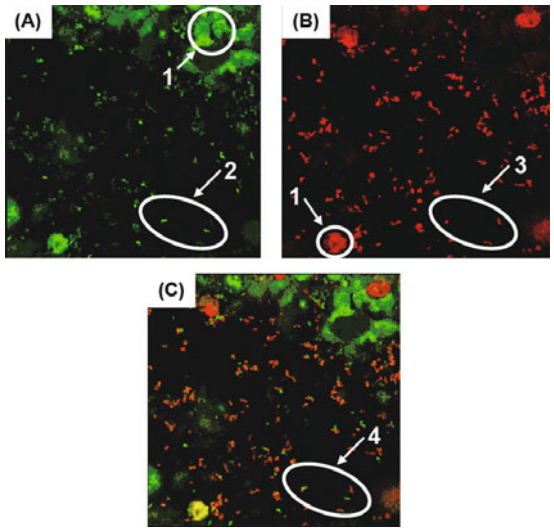

Fig. 3. Observation of the adhesion of live/dead bifidobacterial cells to Caco-2 cells by LSCM (magnification, ×40). (A) the layer for the live bifidobacteria adhered to the Caco-2 cells, (B) the layer for the dead bifidobacteria adhered to the Caco-2 cells, (C) the fitting picture from (A) and (B). 1, the Caco-2 monolayers; 2, live bifidobacterial cells; 3, dead bifidobacterial cells; 4, live and dead bifidobacterial cells.

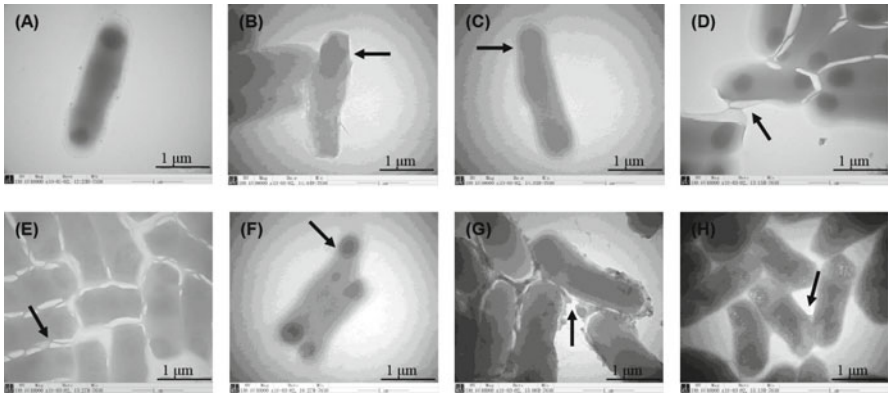

Fig. 4. Observation of the surfaces of *B. animalis* KLDS2.0603 after different treatments (magnification, ×40,000). (A) Control treatment (PBS, pH 7.2), (B) phosphate buffer (pH 2.8) treatment, (C) acetate buffer (pH 4.5) treatment, (D) Trypsin treatment, (E) Pepsin treatment, (F) Proteinase K treatment, (G) LiCl treatment, (H) Sodium Metaperiodate treatment. The arrows indicated the destroyed position on the surface of the bacterial cells.

dobacterial cells were subjected to trypsin, proteinase K, phosphate buffer (pH 2.8), acetate buffer (pH 4.5), and pepsin (97.60%, 97.63%, 98.83%, 99.91%, and 98.78% respectively).

Adhesion of Live/Dead bifidobacteria to Caco-2 cells

According to the instructions of the LIVE/DEAD® BacLight™ bacterial viability kit, the SYTO9 stain generally labels the bacteria with intact membranes as fluorescent green, whereas propidium iodide penetrates only bacteria with damaged membranes and stains fluorescent red. The adhesion ability of live/dead *B. animalis* KLDS2.0603 was determined by this technique. In Fig. 3, the green points were the living bacteria stained by SYTO9, and the red points were the dead bacteria stained by PI. The large green backgrounds with irregular shapes were living Caco-2 cells, and the red ones were dead Caco-2 cells. LSCM examinations confirmed that the live cells could strongly adhere to Caco-2 cells and their intercellular spaces (Fig. 3A). Moreover, the bifidobacterial cells killed by isopropyl alcohol also showed high adhesion to Caco-2 (Fig. 3B), at levels comparable to that of the live untreated cells. The ratio for the adhesion of live and dead cells to Caco-2 cells was about 1:1 for the live and dead bifidobacterial cell mixture (Fig. 3C), which corresponded to the ratio of live/dead cells used in this experiment.

TEM examination of the surface of bifidobacterial cells subjected to certain treatments by TEM

Transmission electron microscopy was used to examine any changes on the surface of *B. animalis* KLDS2.0603. When the cells were subjected to a protease, such as trypsin, pepsin and proteinase K, the surface layers of the cells were digested and destroyed; the appearance of the bacterial cells became fuzzy, and the cells adhered to each other (Figs. 4D, 4E, and 4F). The surfaces of bifidobacterial cells subjected to phosphate buffer (pH 2.8) were rougher than those subjected to acetate buffer (pH 4.5) (Figs. 4B and 4C), which indicated that the composition of the surface of bifidobacteria may be changed by low pH. The treatment with LiCl completely destroyed the surface layer of bifidobacterial cells (Fig. 4G). Although bifidobacterial cells subjected to sodium metaperiodate still maintained an intact morphology, the cell nucleus became clear and the color of the bacterial cells was deeper than that of cells subjected to the control buffer (Figs. 4A and 4H).

Adhesion of bifidobacterial cells subjected to different treatments

The adhesion of *B. animalis* KLDS2.0603 cells that had been subjected to different enzymatic and chemical treatments was observed by LSCM. PBS (pH 7.2) was used as the con-

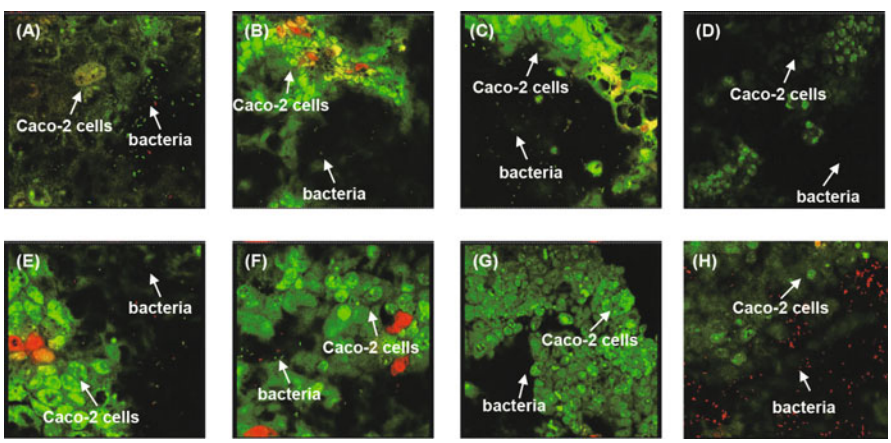

Fig. 5. Observation of the adhesion of *B. animalis* KLDS2.0603 to Caco-2 cells after different treatments by LSCM (magnification, ×40). (A) Control treatment (PBS, pH 7.2), (B) phosphate buffer (pH 2.8) treatment, (C) acetate buffer (pH 4.5) treatment, (D) Trypsin treatment, (E) Pepsin treatment, (F) Proteinase K treatment, (G) LiCl treatment, (H) sodium metaperiodate treatment.

trol buffer for treating bifidobacterial cells (Fig. 5A). The adhesion ability was not affected significantly after the treatment of acetate buffer (pH 4.5) (Fig. 5C); however, it was reduced significantly (Figs. 5D, 5E, and 5F) after treatment with various proteolytic enzymes. The same effect was observed when the cells were treated with phosphate buffer (pH 2.8) (Fig. 5B) and LiCl (Fig. 5G). Although all the bifidobacterial cells were dead following sodium metaperiodate treatment, their adhesion ability did not decrease.

Discussion

The surface layer of bifidobacteria plays the key role in the interaction between bifidobacteria and intestinal epithelial cells. Some papers have shown that components on the bacterial surface may be affected by protease treatment and various conditions (Pérez *et al.*, 1998; Canzi *et al.*, 2005; Frece *et al.*, 2005b; Wang *et al.*, 2010). In the present study, substantial variability in adhesion ability was observed for *B. animalis* KLDS2.0603 subjected to different treatments. The adhesion ability of *B. animalis* KLDS2.0603 was reduced after treatment with any of three proteinases (trypsin, pepsin, and proteinase K), thereby showing that the proteinaceous substances on the cell surface were involved in the adhesion process of *B. animalis* KLDS2.0603. The adhesion ability of *B. animalis* KLDS2.0603 was increased significantly after treatment with sodium metaperiodate (Fig. 1). Carbohydrates on the cell surface would be oxidized by metaperiodate (Kos *et al.*, 2003; Guglielmetti *et al.*, 2009; Zhang *et al.*, 2012); thus, this observation suggested that the adhesion ability of bifidobacteria was not affected by surface carbohydrates. We presume that the surface proteins related to the adhesion ability of bifidobacteria might have been exposed when the carbohydrates were oxidized (Greene and Klaenhammer, 1994). Whatever the mechanism, we have demonstrated that these treatments can alter the adhesive characteristics of bifidobacteria.

Previous studies have reported that some probiotics survive treatments with acid and bile salts (Bezkorovainy, 2001; Ouwehand *et al.*, 2001; Takahashi *et al.*, 2004; Liu *et al.*, 2007). In this study, the survival rate of bifidobacterial cells after treatments with proteolytic enzymes, LiCl and sodium metaperiodate were examined. The survival rate of bifidobacteria was strongly affected by treatments with sodium metaperiodate and LiCl (Fig. 2). Existing reports have shown that the important characteristic of sodium metaperiodate was oxidation and that more than 90% of S-layer proteins from probiotic strains could be extracted by LiCl (Kos *et al.*, 2003). Therefore, whether the adhesion ability of bifidobacteria is affected just by the live/dead state alone needs further study.

The two-color fluorescence assay of bacterial viability is a novel staining technique for determining the live/dead state of cells. The applicability of the commercially available LIVE/DEAD® BacLight™ bacterial viability kit has been evaluated on a wide spectrum of bacteria (Ananta and Knorr, 2004; Panicker *et al.*, 2006; Palencia *et al.*, 2008). In this study, we demonstrated that the adhesion ability of *B. animalis* KLDS 2.0603 was not affected by the live/dead state of the cells (Fig. 3). The results of LSCM were similar to the adhesion ability determined by the Gram staining method (Fig. 1). Compared to the control assay, the total number of bacteria adhering to the Caco-2 cells was decreased when the bacteria were subjected to low pH, trypsin, pepsin, proteinase K, and LiCl. For sodium metaperiodate, which killed all the bifidobacteria, the adhesion ability was not affected significantly (Wang *et al.*, 2010). This indicated that the dead bifidobacterial cells with apparently normal morphology still showed good adhesion ability. The results of this study could help us to further evaluate the possible beneficial effects of dead probiotic cells.

Combining the results of TEM (Fig. 4) and LSCM (Fig. 5), we found that the surface layers of bifidobacterial cells subjected to trypsin, pepsin, proteinase K or LiCl were all destroyed to a certain degree. Although all the cells of bifidobacteria subjected to sodium metaperiodate were oxidized and had deeper color than the cells subjected to the control buffer, the shape of the cells remained intact. This may help explain the strong adhesive ability of *B. animalis* KLDS2.0603 treated by sodium metaperiodate.

In conclusion, our findings indicated that adhesion ability of bifidobacteria was mediated by the components on the cell surface, and the effect of these components on adhesion ability was correlated with the integrity of the cell, but not with the live/dead state of the cell. The cell components responsible for the adhesion ability of bifidobacteria probably were proteins or proteinaceous substances. However, to determine the characteristics of these proteins and the mechanism underlying the adhesion ability of bifidobacteria would need further investigation.

Acknowledgements

Financial support was obtained from the National Natural Science Foundation of China (31171719), Program for Changjiang Scholars and Innovative Research Team in University from China (IRT0959), the National High Technology Research and Development Program of China (863 Program) (2011AA100902).

References

Ananta, E. and Knorr, D. 2004. Evidence on the role of protein biosynthesis in the induction of heat tolerance of *Lactobacillus rhamnosus* GG by pressure pre-treatment. *Int. J. Food Microbiol.* **96**, 307–313.

Bernet, M.F., Brassart, D., Neeser, J.R., and Servin, A.L. 1993. Adhesion of human bifidobacterial strains to cultured human intestinal epithelial cells and inhibition of enteropathogen-cell interactions. *Appl. Environ. Microbiol.* **59**, 4121–4128.

Bezkorovainy, A. 2001. Probiotics: determinants of survival and growth in the gut. *Am. J. Clin. Nutr.* **73**, 399S–405S.

Candela, M., Seibold, G., Vitali, B., Lachenmaier, S., Eikmanns, B.J., and Brigidi, P. 2005. Real-time PCR quantification of bacterial adhesion to Caco-2 cells: competition between bifidobacteria and enteropathogens. *Res. Microbiol.* **156**, 887–895.

Canzi, E., Guglielmetti, S., Mora, D., Tamagnini, I., and Parini, C. 2005. Conditions affecting cell surface properties of human intestinal bifidobacteria. *Antonie van Leeuwenhoek* **88**, 207–219.

Collado, M.C., Meriluoto, J., and Salminen, S. 2007. Development of new probiotics by strain combinations: is it possible to improve the adhesion to intestinal mucus? *J. Dairy Sci.* **90**, 2710–2716.

Collado, M.C., Meriluoto, J., and Salminen, S. 2008. Adhesion and aggregation properties of probiotic and pathogen strains. *Eur. Food Res. Technol.* **226**, 1065–1073.

Deepika, G., Green, R.J., Frazier, R.A., and Charalampopoulos, D. 2009. Effect of growth time on the surface and adhesion properties of *Lactobacillus rhamnosus* GG. *J. Appl. Microbiol.* **107**, 1230–1240.

Del Re, B., Sgorbati, B., Miglioli, M., and Palenzona, D. 2000. Adhesion, autoaggregation and hydrophobicity of 13 strains of *Bifidobacterium longum*. *Lett. Appl. Microbiol.* **31**, 438–442.

FAO/WHO. 2001. Report of a Joint FAO/WHO Expert Group. Health and nutritional properties of probiotics in food including powder milk with live lactic acid bacteria. Food and Agricultural Organization of the United Nations, World Health Organization, Córdoba, 1–4 October (http://www.who.int/foodsafety/publications/fs_management/en/probiotics.pdf).

Fontaine, I.F., Aissi, E.A., and Bouquelet, S.J.-L. 1994. *In vitro* binding of *Bifidobacterium bifidum* DSM 20082 to mucosal glycoproteins and hemagglutinating activity. *Curr. Microbiol.* **28**, 325–330.

Frece, J., Kos, B., Beganović, S., Vuković, S., and Šušković, J. 2005a. *In vivo* testing of functional properties of three selected probiotic strains. *World J. Microbiol. Biotechnol.* **21**, 1401–1408.

Frece, J., Kos, B., Svetec, I.K., Zgaga, Z., Mrša, V., and Šušković, J. 2005b. Importance of S-layer proteins in probiotic activity of *Lactobacillus acidophilus* M92. *J. Appl. Microbiol.* **98**, 285–292.

Greene, J.D. and Klaenhammer, T.R. 1994. Factors involved in adherence of lactobacilli to human Caco-2 cells. *Appl. Environ. Microbiol.* **60**, 4487–4494.

Guglielmetti, S., Tamagnini, I., Minuzzo, M., Arioli, S., Parini, C., Comelli, E., and Mora, D. 2009. Study of the adhesion of *Bifidobacterium bifidum* MIMBb75 to human intestinal cell lines. *Curr. Microbiol.* **59**, 167–172.

Guglielmetti, S., Tamagnini, I., Mora, D., Minuzzo, M., Scarafoni, A., Arioli, S., Hellman, J., Karp, M., and Parini, C. 2008. Implication of an outer surface lipoprotein in adhesion of *Bifidobacterium bifidum* to Caco-2 cells. *Appl. Environ. Microbiol.* **74**, 4695–4702.

Jankowska, A., Laubitz, D., Antushevich, H., Zabielski, R., and Grzesiuk, E. 2008. Competition of *Lactobacillus paracasei* with *Salmonella enterica* for adhesion to Caco-2 cells. *J. Biomed. Biotechnol.* Doi:10.1155/2008/357964.

Kos, B., Šušković, J., Vuković, S., Simpraga, M., Frece, J., and Matosić, S. 2003 Adhesion and aggregation ability of probiotic strain *Lactobacillus acidophilus* M92. *J. Appl. Microbiol.* **94**, 981–987.

Le Blay, G., Fliss, I., and Lacroix, C. 2004. Comparative detection of bacterial adhesion to Caco-2 cells with ELISA, radioactivity and plate count methods. *J. Microbiol. Methods* **59**, 211–221.

Liu, Z., Jiang, Z., Zhou, K., Li, P.L., Liu, G., and Zhang, B. 2007. Screening of bifidobacteria with acquired tolerance to human gastrointestinal tract. *Anaerobe* **13**, 215–219.

Ouwehand, A.C., Tölkkö, S., and Salminen, S. 2001. The effect of digestive enzymes on the adhesion of probiotic bacteria *in vitro*. *J. Food Sci.* **66**, 856–859.

Palencia, P.F., López, P., Corbí, A.L., Peláez, C., and Requena, T. 2008. Probiotic strains: survival under simulated gastrointestinal conditions, *in vitro* adhesion to Caco-2 cells and effect on cytokine secretion. *Eur. Food Res. Technol.* **227**, 1475–1484.

Panicker, G., Aislabie, J., and Bej, A.K. 2006. Analysis of aggregative behavior of *Pseudomonas* sp. 30-3 isolated from Antarctic soil. *Soil Biol. Biochem.* **38**, 3152–3157.

Pérez, P.F., Minnaard, Y., Disalvo, E.A., and De Antoni, G.L. 1998. Surface properties of bifidobacterial strains of human origin. *Appl. Environ. Microbiol.* **64**, 21–26.

Samot, J., Lebreton, J., and Badet, C. 2011. Adherence capacities of oral lactobacilli for potential probiotic purposes. *Anaerobe* **17**, 69–72.

Takahashi, N., Xiao, J.Z., Miyaji, K., Yaeshiima, T., Hiramatsu, K.I., Kokubo, S., and Hosono, A. 2004. Selection of acid tolerant bifidobacteria and evidence for a low-pH-inducible acid tolerance reponse in *Bifidobacterium longum*. *J. Dairy Res.* **71**, 340–345.

Wang, L.Q., Meng, X.C., Zhang, B.R., Wang, Y., and Shang, Y.L. 2010. Influence of cell surface properties on adhesion ability of bifidobacteria. *World J. Microbiol. Biotechnol.* **26**, 1999–2007.

Watanabe, M., Kinoshita, H., Nitta, M., Yukishita, R., Kawai, Y., Kimura, K., Taketomo, N., Yamazaki, Y., Tateno, Y., Miura, K., and *et al.* 2010. Identification of a new adhesion-like protein from *Lactobacillus mucosae* ME-340 with specific affinity to the human blood group A and B antigens. *J. Appl. Micriobiol.* **109**, 927–935.

Zhang, Y.C., Zhang, L.W., Ma, W., Yi, H.X., Yang, X., Du, M., Shan, Y.J., Han, X., and Zhang, L.L. 2012. Screening of probiotic lactobacilli for inhibition of *Shigella sonnei* and the macromolecules involved in inhibition. *Anaerobe* **18**, 498–503.