Aggregation and adhesion properties of 22 *Lactobacillus* strains

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ABSTRACT

In this paper, the autoaggregating, coaggregating, hydrophobicity, and adhering abilities of 22 *Lactobacillus* strains belonging to different species were assessed. No correlation existed between autoaggregation and adhesion of the strains belonging to different species, whereas a positive correlation existed between autoaggregation and adhesion of the strains belonging to the same species. After treating with guanidine HCl, the autoaggregating and adhering abilities of some *Lactobacillus* strains decreased, indicating that surface-bound proteins and other macromolecules played a role in the adhering and autoaggregating abilities. The strains *Lactobacillus plantarum* 20 and 66 had higher adhesion and coaggregation abilities and should be further studied for their probable probiotic properties. Aggregating, coaggregating, and adhering abilities of *Lactobacillus* strains could be used as the preliminary criteria for selecting strains having probiotic potential.

Key words: autoaggregating, coaggregating, hydrophobicity, adhesion

INTRODUCTION

A probiotic is generally defined as “a live microorganism which, when administered in adequate amounts, confers health benefit on the host” (Araya et al., 2002). *Lactobacillus* could exert beneficial effect on humans by inhibiting invasion of pathogens, improving the epithelial barrier function, modulating host immune system, and so on (Saxelin et al., 2005). Many strains of lactobacilli have a variety of health-promoting effects in humans. Adherence and colonization of *Lactobacillus* strains in the gastrointestinal tract is the prerequisite for the strains to exhibit beneficial effect on humans (von Ossowski et al., 2010). The assessment of autoaggregation, coaggregation, hydrophobicity, and adherence to intestinal epithelial cell lines (Caco-2 and HT-29 cells) of *Lactobacillus* strains has been used as

an in vitro method to screen strains having probiotic potential (Kos et al., 2003; Vlková et al., 2008; Tamang et al., 2009; Kirtzalidou et al., 2011).

Human enterocyte-like Caco-2 and HT-29 cell cultures have been used as models to assess the adherence ability of *Lactobacillus* strains to intestinal epithelial cells (Gopal et al., 2001; Servin and Coconnier, 2003), but the methods are time-consuming and expensive. The autoaggregation abilities and hydrophobicity, rather than adherence ability, of *Lactobacillus* strains were assessed for preliminary identification of potentially adherent bacteria (Collado et al., 2007b; Collado et al., 2008; Bao et al., 2010; Chen et al., 2010). It was reported that adherence and autoaggregation of *Lactobacillus* and *Bifidobacteria* strains were strongly related (Del Re et al., 2000). Some probiotic strains can inhibit adherence of pathogenic bacteria to intestinal mucosa either by forming a barrier via autoaggregation or by direct coaggregation with the pathogens (Collado et al., 2007a; Vlková et al., 2008; Ferreira et al., 2011). Lactobacilli that had high autoaggregation ability showed high hydrophobicity (Chen et al., 2010; Nikolic et al., 2010). It was reported that the proteins, glycoproteins, and teichoic and lipoteichoic acids on the cell wall surface of bacteria play important roles in the autoaggregation and hydrophobicity of the strains (Lahtinen et al., 2009; Goh and Klaenhammer, 2010).

Adhesion ability is regarded as an important property of probiotics. When probiotics adhere to the epithelium, they can function stably in the intestine. A correlation between adhesion ability and hydrophobicity has been observed in some lactobacilli (Kos et al., 2003). In this study, we examined the autoaggregation, hydrophobicity, coaggregation, and adhesion ability of 22 lactobacilli strains to preliminary screen some strains having probiotic potential and determined the correlation between cell surface characteristics and adhesion.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions

Lactobacillus strains and *Escherichia coli* O157 were used from our stock culture collection. *Lactobacillus rhamnosus* GG (LGG) was presented as a gift from

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L. Zhang from the Harbin Institute of Technology (Harbin, China). Lactobacilli were cultured anaerobically in de Man, Rogosa, and Sharpe (MRS) broth (Merck KGaA, Darmstadt, Germany) at 37°C for 20 h. *Escherichia coli* O157 was cultured aerobically in brain heart infusion (BHI) broth (Difco Laboratories Inc., Detroit, MI) at 37°C for 20 h.

Caco-2 Cell Culture

The human colonic cell line Caco-2 was provided kindly by X. Chen (Ruijin Hospital, Shanghai, China). The Caco-2 cells were grown routinely in Dulbecco's Modified Eagle Medium (Gibco by Life Technologies Corp., Grand Island, NY), supplemented with 10% (vol/vol) heat-inactivated fetal bovine serum (Sijiqing; Zhejiang Tianhang Biological Technology Co. Ltd., Hangzhou, China) and penicillin (100 U/mL) and streptomycin (100 µg/mL; Pen Strep; Gibco by Life Technologies Corp.) at 37°C in 5% CO₂ atmosphere. The culture medium was replaced every 48 h to maintain the cells.

Autoaggregation and Coaggregation Assays

Autoaggregation and coaggregation assays were carried out according to Collado et al. (2008) with little modification. *Lactobacillus* strains were grown at 37°C for 20 h in MRS broth. The bacteria cells were harvested by centrifugation at 10,000 × *g* for 10 min at 4°C and washed twice with PBS (pH 7.2), and then resuspended in the PBS. Absorbance at a wavelength of 600 nm ($A_{600\text{nm}}$) was adjusted to 0.25 ± 0.05 to standardize the number of bacteria (10^7 – 10^8 cfu/mL). Then, the bacterial suspensions were incubated in 1-mL aliquots at 37°C, which were monitored at different times (0 or 5 h). Autoaggregation percentage was expressed as $[1 - A_t/A_0] \times 100$, where A_t represents the absorbance at time $t = 5$ h and A_0 the absorbance at $t = 0$.

Bacterial suspensions were prepared as described for autoaggregation analysis. Equal volumes of cells (1 mL) of the different probiotic and pathogen strains were mixed and incubated at 37°C without agitation. The $A_{600\text{nm}}$ of the mixtures described above were monitored at different times (0 or 5 h). Absorbance was determined for the mixture and for the bacterial suspensions alone. Coaggregation was calculated as follows: $\{[(A_{\text{pat}} + A_{\text{probio}})/2 - A_{\text{mix}}]/(A_{\text{pat}} + A_{\text{probio}})/2\} \times 100$, where A_{pat} and A_{probio} represent $A_{600\text{nm}}$ of the separate bacterial suspensions in control tubes and A_{mix} represents the absorbance of the mixed bacterial suspension at 5 h.

Bacterial Adhesion to Hydrocarbons

The hydrophobicity of the strains was determined by xylene extraction according to Collado et al. (2008).

Bacterial suspensions were prepared as described above. Absorbance ($A_{600\text{nm}}$) was adjusted to 0.25 ± 0.05 and then an equal volume of xylene was added. The 2-phase system was thoroughly mixed by vortexing for 3 min. The aqueous phase was removed after 1 h of incubation at room temperature and its absorbance at 600 nm was measured. Affinity to hydrocarbons (hydrophobicity) was reported as adhesion percentage according to the formula $[(A_0 - A)/A_0] \times 100$, where A_0 and A are the absorbance before and after extraction with organic solvents respectively.

Adhesion of Bacteria to Caco-2 Cells

The adhesive activity of lactobacilli strains was assessed by using Caco-2 cells as an intestinal epithelial cell model according to Nueno-Palop and Narbad (2011). Caco-2 cells were seeded on 12-well cell culture plates (Greiner Bio-One GmbH, Frickenhausen, Germany) at a concentration of 1×10^5 cells per well. The plates were cultured at 37°C in a humidified atmosphere of 5% CO₂ and 95% air until a confluent monolayer were obtained.

Caco-2 cell monolayers on the 12-well plates were washed twice with PBS (pH 7.2) before the adhesion assay. Bacteria were harvested by centrifugation at 10,000 × *g* for 10 min at 4°C and washed twice with PBS (pH 7.2), and then resuspended in Dulbecco's Modified Eagle Medium (antibiotic-free, fetal bovine serum-free). One milliliter of the bacterial suspension was added to the 12-well plates and incubated for 1 h at 37°C in 5% CO₂ atmosphere. After incubation, each well of the plates was washed 4 times with PBS (pH 7.2) to remove free, nonattached bacterial cells. Then 1 mL of 1% (vol/vol) Triton X-100 was added to each well. Then suspension was stirred to detach the bacterial cells from Caco-2 cell monolayers. Serial dilutions of the suspension were plated onto MRS plates and incubated under anaerobic conditions at 37°C to determine the viable bacterial cell number.

The Effect of Treatment by Guanidine-HCl on the Autoaggregation and Adhesion of the Lactobacilli

All tested strains were harvested by centrifugation at 10,000 × *g* for 10 min at 4°C and washed twice with PBS (pH 7.2). Each of the tested strains was divided into 2 equal parts, one resuspended in 0.5 mL of PBS as control and the other resuspended in 0.5 mL of 4 M guanidine-HCl, and incubated at 37°C for 1 h. Then, the autoaggregation and adhesion of the guanidine-HCl-treated lactobacilli were assessed as described above.

Statistical Analysis

All experiments were performed in triplicate. The data were assessed using ANOVA, with the level of significance at $P < 0.05$. The results are presented as mean $\pm$ standard deviation. Significant divergences among mean values were determined with Duncan's multiple range tests. All statistical analyses were performed using IBM SPSS Statistics 20 software (IBM Corp., Armonk, NY).

RESULTS AND DISCUSSION

Autoaggregation Activity and Hydrophobicity

Twenty *Lactobacillus* strains exhibited autoaggregation ranging from 24.16 to 41.39% after 5 h of incubation at 37°C, as shown in Table 1. We used LGG as the positive control, which showed the highest autoaggregative ability (41.39%).

Cell surface hydrophobicity was measured by xylene extraction. For all the test strains, a significant difference in hydrophobicity was observed, ranging from 16.90 to 96.62%, as shown in Table 1. Most strains showed higher hydrophobicity than LGG (64.14%), whereas *Lactobacillus casei* 94 showed the lowest hydrophobicity (16.90%).

Aggregation plays an important role in the formation of biofilms. In most cases, aggregation ability is related to cell adherence properties, also involving their abil-

ity to survive and persist in the gastrointestinal track (Vlková et al., 2008; Ferreira et al., 2011). A relationship between autoaggregation and adhesion ability has been reported for some bifidobacteria species (Del Re et al., 2000). The hydrophobic nature of the outermost surface of microorganisms has been implicated in the attachment of bacteria to host tissue; this property could confer a competitive advantage, important for bacterial maintenance in the human gastrointestinal tract (Schillinger et al., 2005).

No statistically significant correlations were found between autoaggregation and hydrophobicity of the tested strains in this paper, which belong to different species. This corresponds with the study of Vlková et al. (2008), who reported that hydrophobicity levels of bifidobacteria have no correlation with autoaggregation ability. In our study, significant correlations ($P < 0.01$) existed between autoaggregation and hydrophobicity of the 15 *Lactobacillus plantarum* strains, as shown in Figure 1. The property may be species specific.

Coaggregation Assay of Pathogens with Lactobacilli Strains

The coaggregation ratios are shown in Table 1. All tested strains showed some coaggregation properties with *E. coli* O157:H7. But this property was also strain specific. The strain *Lb. casei* 137 showed the highest coaggregating ability (61%) with *E. coli* O157:H7. The coaggregation ability of LGG was 21.81%.

Table 1. The adhesion ability, autoaggregation, coaggregation, and hydrophobicity of 22 strains of lactobacilli¹

Strain	Adhesion (bacteria/cell)	Autoaggregation at 5 h (%)	Coaggregation at 5 h (%)	Hydrophobicity (%)
<i>Lactobacillus plantarum</i> 13	36.14 $\pm$ 2.34	30.11 $\pm$ 1.24	24.59 $\pm$ 0.47	86.67 $\pm$ 7.55*
<i>Lb. plantarum</i> 20	48.34 $\pm$ 32.12*	31.82 $\pm$ 2.86	27.25 $\pm$ 2.67*	70.98 $\pm$ 6.52
<i>Lb. plantarum</i> 21	40.68 $\pm$ 6.71	29.47 $\pm$ 2.63	25.78 $\pm$ 2.83*	61.82 $\pm$ 15.76
<i>Lb. plantarum</i> 22	32.55 $\pm$ 15.13	30.86 $\pm$ 1.44	24.46 $\pm$ 1.83	83.02 $\pm$ 5.25
<i>Lb. plantarum</i> 23	37.76 $\pm$ 7.77	32.51 $\pm$ 5.50	26.39 $\pm$ 2.23*	82.57 $\pm$ 11.32
<i>Lb. plantarum</i> 27	48.81 $\pm$ 7.29*	33.57 $\pm$ 2.21*	23.35 $\pm$ 1.46	76.42 $\pm$ 1.21
<i>Lb. plantarum</i> 56	46.77 $\pm$ 7.69	29.92 $\pm$ 3.41	22.72 $\pm$ 0.96	62.55 $\pm$ 3.18
<i>Lb. plantarum</i> 57	50.91 $\pm$ 16.98*	30.41 $\pm$ 0.43	23.56 $\pm$ 0.47	64.02 $\pm$ 2.56
<i>Lb. plantarum</i> 66	51.68 $\pm$ 25.66*	33.08 $\pm$ 0.86*	26.72 $\pm$ 1.55*	76.87 $\pm$ 8.94
<i>Lb. plantarum</i> 67	52.88 $\pm$ 25.70*	31.18 $\pm$ 4.26	22.88 $\pm$ 1.02	84.13 $\pm$ 0.56*
<i>Lb. plantarum</i> 68	37.16 $\pm$ 4.58	32.30 $\pm$ 0.04	25.39 $\pm$ 1.44	75.89 $\pm$ 13.50
<i>Lb. plantarum</i> 89	27.25 $\pm$ 1.73	31.04 $\pm$ 4.59	23.38 $\pm$ 1.72	67.77 $\pm$ 7.67
<i>Lb. plantarum</i> 91	39.25 $\pm$ 3.55	29.80 $\pm$ 0.02	24.99 $\pm$ 1.81	86.59 $\pm$ 8.97*
<i>Lactobacillus casei</i> 94	0.00 $\pm$ 0.00	28.52 $\pm$ 11.17	21.62 $\pm$ 1.74	16.90 $\pm$ 8.58
<i>Lb. casei</i> 108	2.58 $\pm$ 0.58	40.94 $\pm$ 6.60*	24.03 $\pm$ 2.59	65.08 $\pm$ 5.16
<i>Lactobacillus rhamnosus</i> 110	3.19 $\pm$ 1.25	26.48 $\pm$ 17.46	21.36 $\pm$ 1.71	96.62 $\pm$ 1.35*
<i>Lb. plantarum</i> 130	31.86 $\pm$ 0.58	24.16 $\pm$ 1.47	21.49 $\pm$ 0.69	26.71 $\pm$ 1.45
<i>Lb. casei</i> 137	1.29 $\pm$ 0.48	33.65 $\pm$ 10.07*	32.16 $\pm$ 1.07*	89.97 $\pm$ 6.31*
<i>Lb. casei</i> 142	0.00 $\pm$ 0.00	30.96 $\pm$ 3.76	22.79 $\pm$ 1.51	82.08 $\pm$ 7.75
<i>Lb. casei</i> 196	21.42 $\pm$ 6.14	35.44 $\pm$ 0.88*	22.81 $\pm$ 1.78	35.79 $\pm$ 5.14
<i>Lb. plantarum</i> f12	23.86 $\pm$ 4.41	24.83 $\pm$ 3.42	25.27 $\pm$ 1.18	45.54 $\pm$ 6.14
<i>Lb. rhamnosus</i> GG	9.66 $\pm$ 2.54	41.39 $\pm$ 3.30*	21.81 $\pm$ 1.26	64.14 $\pm$ 20.26

¹Values are means of 3 replicates $\pm$ SD.

*Significant difference ($P < 0.05$) among all *Lactobacillus* strains within the same column.

Coaggregation may play an important role in eliminating pathogens from the gastrointestinal tract (Todorov et al., 2008). *Lactobacillus* strains can form a barrier that prevent colonization by pathogenic bacteria through coaggregation (Ferreira et al., 2011). Coaggregation with a potential pathogen allows the probiotic strain to produce antimicrobial substances in very close proximity to them, which may inhibit the growth of pathogenic strains in the gastrointestinal tract (Reid et al., 1988). It was reported that autoaggregation properties together with coaggregation ability with potential pathogens can be used for preliminary selection of probiotic bacteria (Collado et al., 2007a; Vlková et al., 2008; Bernardeau et al., 2009; Ferreira et al., 2011). Autoaggregation and coaggregation are important in the formation of biofilms to protect the host from colonization by pathogens (Ocaña and Nader-Macias, 2002).

Adhesion of Bacteria to Caco-2 Cells

The adhering ability of 20 strains was examined in vitro. The number of strains adhering to Caco-2 cells varied from 0 to 52.88 cfu/cell, as shown in Table 1. Whereas LGG showed an adhering ability of 9 cfu/cell, lower than that of most of the strains, *Lb. casei* 142 and *Lb. casei* 94 were nonadhesive, and *Lb. plantarum* 20, *Lb. plantarum* 66, *Lb. plantarum* 57, *Lb. plantarum* 67, and *Lb. plantarum* 27 showed strong adhering ability. The strains *Lb. plantarum* 20 and *Lb. plantarum* 66 also had higher coaggregation ability; they may have probiotic potential. Further studies should be carried out.

Researchers have found good correlations between adhesion to human intestinal mucus and to Caco-2 models (Gueimonde et al., 2006). Rosenfeldt et al. (2003) reported that probiotic strains with higher adhering ability could adhere to colonic mucosa. Adhesion is considered an important property of probiotic lactic acid bacteria able to colonize the gastrointestinal tract (Holzapfel et al., 1998). In our research, LGG had lower adherence to Caco-2 cells compared with other lactobacilli, which was in accordance with findings of other researchers (Gopal et al., 2001). But LGG had desirable probiotic functions. The in vitro adhesion ability of *Lactobacillus* (adhering to Caco-2 or HT-29 cells) was not the unique criterion for selecting probiotics.

In some lactobacilli, a correlation between adhesion ability and hydrophobicity has been observed (Ehrmann et al., 2002). In our study, no correlation ($P > 0.05$) existed between hydrophobicity and adhesion of the tested strains. *Lactobacillus rhamnosus* 110 and *Lb. casei* 137 with high hydrophobicity showed low adhesion ability. The results were in accordance with the results of other studies (Schillinger et al., 2005;

Figure 1. Relationship between autoaggregation (%) ability and hydrophobicity (%) of *Lactobacillus plantarum* strains.

Botes et al., 2008; Mathara et al., 2008). The adhesion ability to Caco-2 cells of 15 *Lb. plantarum* strains had positive correlation with the autoaggregation ability of the strains ($P < 0.01$), as shown in Figure 2. Among the strains, including *Lb. casei* 94, *Lb. casei* 108, *Lb. rhamnosus* 110, *Lb. casei* 137, *Lb. casei* 142, *Lb. casei* 196, and LGG, the adhesion ability of the strains had no correlation with aggregation and hydrophobicity of the strains. Our results showed that the adhering ability and autoaggregation ability of *Lactobacillus* strains belonging to different species had no correlation. The correlation between the adhering ability and autoaggregation ability of *Lactobacillus* could be species specific, which would indicate that among different species of *Lactobacillus* strains, selecting *Lactobacillus* strains with higher adhering ability according to the aggregation ability is not a desirable method.

When the strains were treated with guanidine-HCl, the number of bacterial cells adhering to Caco-2 and the autoaggregation ability of the bacteria decreased, as shown in Figures 3 and 4. The results indicated that the surface-bound protein played a partial role in the adhering and autoaggregating abilities of the strains, which is consistent with previous reports (Nikolic et al., 2010). After treatment with guanidine-HCl, the strains 130, 196, 57, and f12 still adhered to Caco-2 cells, which indicates that other molecules on the bacterial cell surface of the strains had affinity to Caco-2 cells. Ramiah et al. (2008) reported that factors other than surface-bound proteins play a role in adhesion. The proteins, glycoproteins, and teichoic and lipoteichoic acids on the cell wall surface of bacteria contribute to the adhesion, autoaggregation abilities, and hydrophobicity of the

Figure 2. Relationship between autoaggregation (%) and adhesion (%) ability of *Lactobacillus plantarum* strains.

strains (Lahtinen et al., 2009; Goh and Klaenhammer, 2010; Darilmaz and Beyatli, 2012). The macromolecules involved in adhesion will be further studied.

Other researchers have indicated that autoaggregation abilities and hydrophobicity are tools for selecting strains having good adhering ability (Collado et al., 2007b, 2008; Bao et al., 2010; Chen et al., 2010), but they did not provide data about the adhering ability of

Figure 3. Comparison of adhesion ability of *Lactobacillus* strains before and after guanidine-HCl treatment. * indicates significant difference ($P < 0.05$) between the guanidine-HCl treated strain and the untreated same strain. The values represent the averages of all 3 experimental replicates, and the error bars represent the SD. 130 = *Lactobacillus plantarum* 130; 196 = *Lactobacillus casei* 196; 23 = *Lb. plantarum* 23; 57 = *Lb. plantarum* 57; 66 = *Lb. plantarum* 66; f12 = *Lb. plantarum* f12; LGG = *Lactobacillus rhamnosus* GG.

Figure 4. Comparison of autoaggregating ability of *Lactobacillus* strains before and after guanidine-HCl treatment. Asterisk (*) indicates significant difference ($P < 0.05$) between the guanidine-HCl treated strain and the same untreated strain. The values represent the averages of all 3 experimental replicates, and the error bars represent the SD. 130 = *Lactobacillus plantarum* 130; 196 = *Lactobacillus casei* 196; 23 = *Lb. plantarum* 23; 57 = *Lb. plantarum* 57; 66 = *Lb. plantarum* 66; f12 = *Lb. plantarum* f12; LGG = *Lactobacillus rhamnosus* GG.

their lactobacilli and the correlation between adhering ability and autoaggregation ability.

CONCLUSIONS

Adhering ability and autoaggregating ability of the *Lactobacillus* strains belonging to the same species had positive correlation; no correlation existed between hydrophobicity and adhesion of the tested strains. Therefore, *Lactobacillus* strains belonging to same species having higher adhering ability could be selected according to their autoaggregating ability. The relationship among adhesion, autoaggregation, coaggregation, hydrophobicity, and the cell surface properties of lactobacilli should be further studied.

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