Original Research Article

Aggregation and adhesion abilities of 18 lactic acid bacteria strains isolated from traditional fermented food

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INTRODUCTION

LABs (Lactic acid bacteria) are normal inhabitants of human gastrointestinal tract and play a quite important role in the maintenance of the colonic microbial ecosystem (Kirtzalidou et al., 2011). Lactic acid bacteria could exert beneficial effect on humans due to their potential health-promoting properties, such as inhibition on invasion of pathogens, improvement of the epithelial barrier function, and so on (Saxelin et al., 2005). Adhesion and colonization of lactic acid bacteria strains in the gastrointestinal tract is considered one of the main criteria for the selection of potential probiotics, as it may increase their persistence in the intestine and thus allow their exertion of probiotic effects (Kolida et al., 2006; von Ossowski et al., 2010).

Since it is difficult to investigate bacterial adherence in vivo, adhesion has been studied using human intestinal cell lines as in vitro models for intestinal epithelium (Tuomola and Salminen, 1998). Human enterocyte-like Caco-2 cell line which was originally isolated from a human colon adenocarcinoma (Cell, 1983; Greene and Klaenhammer, 1994) has been used as a model to assess the adherence ability of lactic acid bacteria strains to intestinal epithelial cells (Pan et al., 2009; Servin and Coconnier, 2003). It was reported that adhesion ability and autoaggregation of lactic acid bacteria and bifidobacteria strains were significantly related (Del Re et al., 2000). Researches had found that some lactic acid bacteria can prevent adherence of pathogenic bacteria to intestinal mucosa either by forming a barrier via autoaggregation or by coaggregation with the pathogens (Collado et al., 2007; Vlkov et al., 2008). Lactobacilli with high autoaggregation ability showed high hydrophobicity (Chen et al., 2010; Nikolic et al., 2010). Previous studies indicated a correlation between adhesion ability and hydrophobicity of some lactobacilli (Wadstrohm et al., 1987; Xu et al., 2009). Mainly cell surface-associated

Bacterial aggregation and hydrophobicity of lactic acid bacteria strains isolated from Chinese traditional fermented food were performed in order to assess a correlation with their adhesion abilities by using intestinal Caco-2 cell line in vitro model. In this study, no correlation existed between hydrophobicity/autoaggregation and adhesion of the strains belonging to different species, whereas a positive correlation (P < 0.01) existed between hydrophobicity/autoaggregation and adhesion of the strains belonging to the same species. When treated with 5 M LiCl, the autoaggregation and adhesion ability of some lactic acid bacteria strains decreased, indicating that surface-bound proteins and other macromolecules were involved in the adhesion and autoaggregation abilities. Hydrophobicity, autoaggregation, and coaggregation abilities of lactic acid bacteria strains could be used as the preliminary criteria for selecting strains with high adhesion ability.

Key words: adhesion, autoaggregation, co-aggregation, hydrophobicity.
proteins are involved in aggregation and adhesion although many other factors existed like teichoic, lipoteichoic acids, and polysaccharide (Goh and Klaenhammer, 2010). Considering economic and time cost, the autoaggregation abilities and hydrophobicity, rather than adherence ability of lactic acid bacteria strains were assessed as a prerequisite for potentially adherent bacteria (Bao et al., 2010; Chen et al., 2010).

Hydrophobicity, autoaggregation, coaggregation, and adhesion ability are phenotypic traits for the screening potential probiotic strain (Kos et al., 2003; Tamang et al., 2009). In this study, the hydrophobicity, autoaggregation, coaggregation, and adhesion ability of 18 lactic acid bacteria strains isolated from Chinese traditional fermented food were evaluated to assess the correlation between cell surface characteristics and adhesion. The other aim of this study is to evaluate whether bacterial aggregation abilities and hydrophobicity of lactic acid bacteria strains could be used as a preliminary assessment on their adhesion properties.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions

Bacterial strains used in this study were *Lactobacillus fermentum* 12-2, *L. fermentum* 1-2, *L. fermentum* GA4, *L. fermentum* GS7, *L. fermentum* L9-1, *L. fermentum* 9sh, *L. fermentum* AB4, *Lactococcus lactis* 1, *L. lactis* 3, *L. lactis* 5, *L. lactis* 8, *L. lactis* 11, *L. lactis* 136, *L. lactis* K10, *L. lactis* 134, *L. lactis* KN, *Enterococcus faecalis* J2, *E. faecalis* 5-5 (listed in Table 1), which were isolated from Chinese traditional fermented food, such as pickled vegetable, koumiss from Xinjiang, tibetan kefir grains and so on. Lactic acid bacteria strains were grown in MRS (Merck GmbH, Darmstadt, Germany) at 37 °C for 20 h. The pathogenic bacterial strain used in this study was *Salmonella* sp., provided by Nanjing Institute of Supervision & Testing on Product Quality (Jiangsu, China), which was cultivated in Luria-Bertani (LB) medium at 37 °C for 20 h.

Cell culture

The human colon adenocarcinoma Caco-2 cell lines were purchased from the Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, China). Caco-2 cells were grown in Dulbecco’s modified Eagle’s medium (DMEM; HyClone, Laboratories Inc., Logan, UT, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS; HyClone), L-glutamine (2 mmol L⁻¹), penicillin (100 µL⁻¹), and streptomycin (100 mg mL⁻¹) in an incubator with 95% (v/v) humidified air and 5% (v/v) CO₂ at 37°C. The culture medium was replaced every 48 h to maintain the cells and the cells were subcultured at 80% confluence every week.

Bacterial adhesion to hydrocarbons

The hydrophobicity of the bacterial strains was performed by xylene extraction according to Collado et al. (2008). Lactic acid bacteria strains were cultivated in MRS broth at 37°C for 20 h. The bacterial cells were harvested by centrifugation at 10,000 × g for 10 min at 4°C and washed twice with phosphate-buffered saline (PBS; pH 7.2), and then re-suspended in the PBS (pH 7.2). The absorbance at 600 nm (OD₆₀₀) was adjusted to 0.25 ± 0.05 to standardize the number of bacteria (approximately 10⁸ cfu/mL). Then an equal volume of xylene was added. The 2-phase system was thoroughly mixed by a vortex for 4 min. The aqueous phase was removed after 1 h of incubation at room temperature and its absorbance at 600 nm was measured. The affinity of the bacterial strains to hydrocarbons (hydrophobicity) was reported as adhesion percentage according to the formula: \[
\left(\frac{A_o - A}{A_o}\right) \times 100
\]
where A₀ and A are the absorbance before and after extraction with organic solvents, respectively.

Autoaggregation and coaggregation assays

Autoaggregation and coaggregation assays were carried out according to Collado et al. (2008) and Tuo et al. (2013) with some modifications. Bacterial suspensions were prepared as described above. Same treatment was done with the pathogen strain to get pathogen suspension. For autoaggregation assay, the absorbance at 600 nm of cell suspensions (4 mL) incubated at 37°C was monitored with spectrophotometer for 5 h at different time (0 and 5 h). Autoaggregation percentage was expressed as \[
\left[1 - \frac{A_t}{A_0}\right] \times 100
\]
where A₀ represents the absorbance at time t = 5 h and Aₜ the absorbance at time t = 0 h.

For coaggregation assay, 2 mL of each bacterial suspension and pathogen suspension were mixed and incubated at 37°C without agitation. The absorbance of the mixtures described above was monitored at different time (0 and 5 h). Absorbance was determined for the bacterial suspensions alone. Coaggregation percentage was calculated as follows:

\[
1 - \frac{Amix}{A_{salm} + A_{probio}} \times 100
\]

Adhesion assay

Ability of the lactic acid bacteria to adhere to the human
The adhesion assay was conducted at 37°C for 20 h. All experiments were carried out in triplicate. The data were assessed using ANOVA with the level of significance at P < 0.05. The results are presented as mean values ± standard deviation. Significant divergences among mean values were determined with Duncan’s multiple range tests (Tuo et al., 2013). All statistical analyses were performed with SPSS 11.0 software (IBM Corp., Armonk, NY).

RESULTS

Hydrophobicity and autoaggregation activity

The bacterial cell surface hydrophobicity was measured by xylene extraction. The results are shown in Table 1. Eighteen strains showed a significant difference in hydrophobicity ranging from 0.86% to 98.78% (P < 0.05). E. faecalis J2 showed the highest hydrophobicity (98.78%), while L. fermentum GA4 showed the lowest hydrophobicity (0.86%).

<table>
<thead>
<tr>
<th>Strain</th>
<th>Hydrophobicity(%)</th>
<th>Autoaggregation(%)</th>
<th>Coaggregation(%)</th>
<th>Adhesion(CFU /cell)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lactococcus fermentum 12-2</td>
<td>1.24 ± 0.31 F</td>
<td>8.99 ± 0.47 H</td>
<td>6.24 ± 0.12 O</td>
<td>48.75 ± 2.40 D</td>
</tr>
<tr>
<td>L. fermentum 1-2</td>
<td>44.26 ± 2.49 M</td>
<td>23.32 ± 2.21 A</td>
<td>8.07 ± 0.24 O</td>
<td>108.44 ± 20.38 B</td>
</tr>
<tr>
<td>L. fermentum GA4</td>
<td>0.86 ± 0.26 R</td>
<td>8.04 ± 0.67 P</td>
<td>6.02 ± 0.39 Q</td>
<td>14.38 ± 1.12 R</td>
</tr>
<tr>
<td>L. fermentum GS7</td>
<td>25.59 ± 2.45 O</td>
<td>15.86 ± 1.47 P</td>
<td>12.42 ± 1.02 M</td>
<td>48.75 ± 3.28 G</td>
</tr>
<tr>
<td>L. fermentum L9-1</td>
<td>63.15 ± 0.57 I</td>
<td>14.34 ± 3.92 G</td>
<td>16.99 ± 0.44 F</td>
<td>156.22 ± 15.48 A</td>
</tr>
<tr>
<td>L. fermentum 9sh</td>
<td>59.70 ± 1.78 K</td>
<td>19.68 ± 2.01 C</td>
<td>29.54 ± 2.37 A</td>
<td>84.43 ± 8.42 K</td>
</tr>
<tr>
<td>L. fermentum AB4</td>
<td>38.72 ± 2.01 N</td>
<td>18.64 ± 2.11 D</td>
<td>19.45 ± 1.03 B</td>
<td>75.05 ± 14.24 F</td>
</tr>
<tr>
<td>Lactococcus lactis 1</td>
<td>62.03 ± 0.43 I</td>
<td>5.92 ± 0.34 P</td>
<td>16.07 ± 1.24 K</td>
<td>30.02 ± 1.22 M</td>
</tr>
<tr>
<td>L. lactis 3</td>
<td>71.64 ± 0.14 F</td>
<td>6.58 ± 0.42 M</td>
<td>13.28 ± 0.43 L</td>
<td>36.89 ± 1.86 E</td>
</tr>
<tr>
<td>L. lactis 5</td>
<td>75.96 ± 4.01 G</td>
<td>6.86 ± 0.32 L</td>
<td>17.02 ± 0.25 K</td>
<td>37.22 ± 2.02 H</td>
</tr>
<tr>
<td>L. lactis 8</td>
<td>63.88 ± 0.24 H</td>
<td>5.98 ± 0.29 P</td>
<td>16.98 ± 0.37 G</td>
<td>30.00 ± 1.48 N</td>
</tr>
<tr>
<td>L. lactis 11</td>
<td>68.24 ± 0.32 G</td>
<td>6.24 ± 0.26 O</td>
<td>16.84 ± 0.12 H</td>
<td>36.82 ± 1.98 B</td>
</tr>
<tr>
<td>L. lactis 136</td>
<td>70.21 ± 1.09 F</td>
<td>6.46 ± 0.31 N</td>
<td>19.17 ± 0.03 C</td>
<td>32.14 ± 2.26 C</td>
</tr>
<tr>
<td>L. lactis K10</td>
<td>97.29 ± 2.23 B</td>
<td>12.49 ± 1.22 H</td>
<td>16.61 ± 0.08 B</td>
<td>104.46 ± 15.87 C</td>
</tr>
<tr>
<td>L. lactis 134</td>
<td>64.12 ± 1.09 H</td>
<td>5.98 ± 0.34 P</td>
<td>17.33 ± 1.14 D</td>
<td>30.96 ± 2.14 M</td>
</tr>
<tr>
<td>L. lactis KN</td>
<td>79.84 ± 0.46 C</td>
<td>7.66 ± 0.44 K</td>
<td>16.78 ± 0.25 L</td>
<td>32.96 ± 1.88 K</td>
</tr>
<tr>
<td>Enterococcus faecalis J2</td>
<td>98.78 ± 0.24 A</td>
<td>16.46 ± 1.98 B</td>
<td>8.79 ± 0.14 N</td>
<td>21.58 ± 1.64 O</td>
</tr>
<tr>
<td>E. faecalis 5-5</td>
<td>49.68 ± 0.31 L</td>
<td>22.80 ± 2.09 B</td>
<td>5.15 ± 0.23 R</td>
<td>104.13 ± 20.08 D</td>
</tr>
</tbody>
</table>

1 Data are mean values ± SD (n = 3).
2-9 Significant difference (P < 0.05) among all Lactic acid bacteria strains within the same column.
About 66.67% of the tested strains showed the hydrophobicity above 50%. The hydrophobicity of *L. lactis* strains and *E. faecalis* J2 were higher than all the *Lactobacillus fermentum* strains. While the results also suggested that different *L. lactis* strains possessed different hydrophobicity.

Eighteen lactic acid bacteria strains tested showed autoaggregation ranging from 5.92% to 23.32% after 5 h of incubation at 37°C (Table 1). Except *L. lactis* K10, all the *L. lactis* strains tested had lower autoaggregation ability than the other strains. *L. fermentum* 1-2 had the strongest autoaggregation while *L. lactis* 1 had the poorest autoaggregation.

It was noted that there were no significant correlation between autoaggregation and hydrophobicity of the 18 tested strains (Table 1 and Figure 1). *L. fermentum* L9-1, which had the highest adhesion to Caco-2 cells (156.22 cfu/cell) showed low autoaggregation ability. While significant correlation between autoaggregation and hydrophobicity were observed among the 9 *Lactococcus lactis* strains, since the correlation coefficient value could reach to 0.953 (P < 0.01). It was observed that strains with higher autoaggregation showed higher hydrophobicity. *L. lactis* K10 which had the strongest autoaggregation among *Lactococcus lactis* strains showed the highest hydrophobicity, while other *L. lactis* strains with low autoaggregation showed poor hydrophobicity.

**Figure 1:** Relationship between autoaggregation ability (%) and hydrophobicity (%) of the strains

**Coaggregation assay of pathogen with lactic acid bacteria strains**

The coaggregation ratios are shown in Table 1. The significant differences for coaggregation existed among the strains. All lactic acid bacteria strains tested in this study showed some coaggregation properties with *Salmonella* sp. ranging from 5.15% to 29.54%, indicating strain-specific characteristics. The strain *L. fermentum* 9sh showed the highest coaggregation ability (29.54%) with *Salmonella* sp., followed by the strain *L. fermentum* AB4 (19.45%). *E. faecalis* 5-5 showed the lowest coaggregation ability. Among the 18 strains, 66.67% of strains indicated coaggregation above 15%, and only 11.11% of strains had coaggregation below 10%.

**Adhesion of bacteria to Caco-2 cells**

All lactic acid bacteria strains tested were able to adhere to Caco-2 cells (Table 1). The number of strains adhering to Caco-2 cells varied from 14.38 to 156.22 cfu/cell. Specific adhesive ability also existed among different strains, since the eighteen strains showed different levels of adhesion to Caco-2 cells. About 22.22% of them were adhered to 100-160 bacteria numbers/cell, 66.67% of them adhered to 20-50 bacteria numbers/cell, and 11.11% below 20 bacteria numbers/cell. *L. fermentum* L9-1 and *L. lactis* K10 showed...
not only higher adhering ability but also higher coaggregation ability, so they may have probiotic potential. Further researches should be carried out to access their antimicrobial activity and protective properties.

Interestingly, no significant correlations were found between autoaggregation and adhesion of the 18 strains (Figure 2), while the adhesion ability of 9 *L. lactis* strains had positive correlation with the autoaggregation ability of the strains ($P < 0.01$). It was seen that strains K10 and KN with high autoaggregation 12.49% and 7.66% had strong abilities to adhere to the human intestinal cell-line Caco-2, while other *L. lactis* strains with low autoaggregation exhibited poor adhesion.

**The effect of treatment by LiCl on the autoaggregation and adhesion of the lactic acid bacteria strains**

The results of the effect of treatment by 5M LiCl on the autoaggregation and adhesion of the lactic acid bacteria strains are shown in Figure 3 and Figure 4. When treated with 5M LiCl, the number of bacterial cells adhering to Caco-2 and the autoaggregation of the lactic acid bacteria strains tested decreased in different degrees. The results suggested that the surface-related proteins played a partial role in the adhering and autoaggregation abilities of the strains. The adhesion of *L. lactis*3, *L. lactis* K10, *L. lactis* KN, and *E. faecalis* J2 did not exist after treated with LiCl, while other strains still showed adhesion to the Caco-2 cells with different levels, indicting other factors involved in the adhesion ability.

**DISCUSSIONS**

Aggregation ability has been suggested to be an important property of many bacterial strains used as probiotics, which plays an important role in the formation of biofilms to protect the host from colonization by pathogens (An et al., 2000). Lactic acid bacteria with aggregation ability and hydrophobicity cell surface could be more capable to adhere to intestinal cells. Previous research found that *Lactobacillus crispatus* adhering better to Caco-2 cells than its non-aggregation mutant (Oca and Macias, 2001). In addition, the key role of hydrophobic interactions in bacterial adhesion is well established since important aspects of microbial behavior are controlled by physicochemical properties of the cell wall (Van Loosdrecht et al., 1987). In this study, significant correlations ($P < 0.01$) were found between autoaggregation and hydrophobicity of the 9 tested *L. lactis* strains, as shown in Figure 1. This is in accordance with the researches of Nikolic et al. (2010) who had reported that autoaggregating strains showed high autoaggregation and adhesion ability.
**Figure 3** Comparison of autoaggregation ability of lactic acid bacteria strains before and after 5 M LiCl treatment. The data were mean values of 3 separate experiments, and the error bars represent the SD.


**Figure 4** Comparison of adhesion ability of lactic acid bacteria strains before and after 5 M LiCl treatment. The data were mean values of 3 separate experiments, and the error bars represent the SD.

hydrophobic nature of the cell surface and Tuo et al. (2013) who had reported that significant correlation existed between autoaggregation and hydrophobicity of 15 Lactobacillus plantarum strains.

Coaggregation of bacterial strains plays a significant role in some ecological niches, especially in the human gut. It was reported that coaggregation abilities may allow lactic acid bacteria strains inhibit the growth of pathogenic strains in the gastrointestinal and urogenital tracts in a very close proximity (Botes et al., 2008). Moreover, lactic acid bacteria strains could control a microenvironment around the pathogens and increase the concentration of excreted antimicrobial substances in the process of coaggregating (Kaewnopparat et al., 2013).

Adhesion ability is of significance in promoting probiotic bacteria to colonize the gastrointestinal tract and confer a healthy benefit to the host. It was noted that no significant correlations were found between adhesion and autoaggregation of the 18 lactic acid bacteria strains belonging to different species tested in this study (Table 1), which corresponded to the research of Cayuela et al. (2014). While positive correlation between adhesion and autoaggregation were found among 9 Lactococcus lactis strains. Previously, a correlation between adhesion ability and hydrophobicity had been observed in some lactobacilli according to Ehrmann et al. (2002). It indicated that the correlation between the adhering ability and autoaggregation ability of lactic acid bacteria could be strain-specific, thus it indicated that among different species of lactic acid bacteria strains, selecting lactic acid bacteria strains with higher adhering ability according to the aggregation ability is not a desirable method.

Adhesion is a complex process including non-specific and specific ligand-receptor mechanisms (Cayuela et al., 2014). Factors such as the proteins, glycoproteins, teichoic, and lipoteichoic acids on the cell wall surface of bacteria were involved in the adhesion, autoaggregation and hydrophobicity abilities (Ramiah et al., 2008). In the past years, LiCl was usually used to remove the surface layer proteins (s-layer proteins) which existed in the paracrystalline layer outside the bacterial cell wall and responsible to the adhesive properties (Ren et al., 2012). In this study, the decrease of autoaggregation and adhesion of the strains after treated with LiCl which was used to remove s-layer protein (Zhang et al., 2013), suggested that the surface-related proteins played a partial role in the adhering and autoaggregation abilities of the strains, which was in accordance with the findings of previous researches (Nikolic et al., 2010; Tuo et al., 2013). While 14 strains (except L. lactis3, L. lactis K10, L. lactis KN, and E. faecalis J2) still adhered to the Caco-2 cells with different extent, indicating that there are other molecules beside surface-bound proteins had affinity to Caco-2 cells. Further researches should be studied for the assessment of the macromolecules involved in the adhesion.

In vitro results of bacteria adhesion are difficult to extrapolate to in vivo conditions, as different elements existed in the dynamic environment of the gastrointestinal tract are likely to modify the bacteria adhesion (Lebeer et al., 2008). However, in vitro experiments are essential to understand the mechanisms of adhesion, and they provide significant information about strain differences. Thus, the results above indicated the requisite for a case-by-case assessment to select new potential probiotic strains and provided a method for screening L. lactis with high ability to adhere to intestinal epithelial cells.

In summary, these results suggest that aggregation, hydrophobicity, and adhesion of lactic acid bacteria strains are strain-specific and the correlation between autoaggregation and hydrophobicity, autoaggregation and adhesion are positive among the 9 L. lactis strains rather than the 18 strains belonging to different species tested. Therefore, bacterial aggregation and hydrophobicity can be used as a preliminary screening to assess their adhesion properties. Moreover, surface-bound proteins may not be the only factor involved in the adhesion of strains, but they can be one of the criteria to bear in mind for understanding the complex interactions of bacteria with the host gut. Further research will be performed to evaluate remaining attributes related to adhesion.

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