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Adhesive ability means inhibition activities for lactobacillus against pathogens and S-layer protein plays an important role in adhesion

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ABSTRACT

Eighty-five strains of lactobacillus were isolated from the pig intestine and identified by sequencing analysis based on 16S rRNA gene, from which five lactobacillus strains with high adhesive ability were selected. The inhibition ability of the five lactobacillus strains with or without S-layer proteins against adherence of Escherichia coli K88 and Salmonella enteritidis 50335 to Caco-2 was evaluated in vitro with Lactobacillus rhamnosus GG strain (LGG) as a positive control. In addition, tolerance of lactobacilli to heat, acid, bile, Zn2+ and Cu2+ were assessed. All five selected strains, Lactobacillus salivarius ZJ614 (JN981856), Lactobacillus reuteri ZJ616 (JN981858), L. reuteri ZJ617 (JN981859), L. reuteri ZJ621 (JN981863) and L. reuteri ZJ623 (JN981865), showed inhibition against the two pathogens, E. coli K88 and S. enteritidis 50335. L. reuteri ZJ621 showed higher inhibition ability than the others to S. enteritidis 50335 (P < 0.05). Sodium dodecyl sulfate-Polyacrylamide gel electrophoresis (SDS-PAGE) analysis indicated that all five strains had abundant bands with molecular weight ranging from 34 to 130 KDa as well as had a common band of approximately 42 KDa. After treatment with 5 M LiCl to remove S-layer protein, the inhibition strains had abundant bands with molecular weight ranging from 34 to 130 KDa as well as had a common band of approximately 42 KDa. Higher adhesive ability means higher inhibition activity for lactobacillus against pathogen, in which S-layer proteins plays an important role.

1. Introduction

Lactobacillus can confer a health benefit on the host under various conditions [1]. The lactobacillus species inhabiting in animals as well as human play an important role in the physiology of their host, including the digestion and assimilation of nutrients [2], protection against the colonization of pathogens [3,4], modulation of immune responses [5,6], regulation of fat storage [7] and beneficial effects on the intestinal microbial balance of host. Wine et al. [8] found that Lactobacillus helveticus prevented Camplylobacter jejuni from invading intestinal epithelium of human. The Lactobacillus stains rhamnosus, acidophilus, casei shirota, fermentum and plantarum inhibited the adhesion of pathogens Bac teroides vulgatus, Clostridium histolyticum, Clostridium difficile, Staphylococcus aureus and Enterobacter aerogenes to intestinal mucus [9]. Although commensal Lactobacillus reuteri and Lactobacillus salivarius showed beneficial effects on animal health [10,11], the inhibition ability against pathogen adhesion was less studied. In addition, the mechanism of adhesion and inhibition against pathogens remains to be elucidated.

Adhesion to intestinal epithelial cell is considered to be the first step for the lactobacillus strains performing beneficial effects on the health of host, and high adhesive ability can promote the gut residence time of lactobacillus, exclusion of pathogens, and protection of epithelial cells [12,13]. The survival and persistence time of lactobacillus in gastrointestinal tracts are extremely important. Therefore, adhesion ability may be tested as a standard for choosing lactobacillus. The adhesion mechanism of lactobacillus is complicated and controversial, and a common theory is that adhesion is a specific interaction between bacterial surface components and their receptors on the host epithelial cells. Many factors are involved in the adhesion of lactobacillus to host epithelial cells such as cell surface component lipoteichoic acid [14], surface layer protein (S-layer protein, Slp) [4,15], peptidoglycan [16] and cell autoaggregation ability [17]. S-layer proteins of lactobacilli have
been commonly suggested to be involved in the adherence of lactobacilli, although not all lactobacilli have an S-layer protein [15,18,19].

The proteinaceous surface components mediating bacterial adhesion to intestinal mucosa and epithelial cells have been demonstrated for many lactobacillus species [20–22]. S-layer proteins are regular structure covering the cell surface and are composed of monomeric protein units known as the S-protein. S-layer proteins of lactobacillus are verified to play an important role in inhibiting pathogens. S-layer proteins extracted from Lactobacillus crispatus strain ZJ001 or Lactobacillus helveticus were identified to be able to inhibit the adhesion of Salmonella typhimurium and Escherichia coli O157:H7 [4,15]. After removal or disruption of the surface proteins from lactobacillus species, the ability of adhesion and inhibition against pathogens of lactobacillus decreased [15,23]. Although many S-layer proteins of lactobacilli have been studied, their functions in terms of adhesion to intestinal mucosa and epithelial cells have been poorly characterized, especially the S-layer proteins from L. reuteri and salivarius strains.

The aim of this study was to investigate the inhibition of adhesion of pathogens to intestinal epithelial cells by lactobacilli with higher adhesive ability isolated from porcine intestinal tract. The function of the S-layer proteins mediating the adhesion of L. reuteri and salivarius strains to Caco-2 cells was examined. It is expected to verify if higher adhesive ability means higher inhibition activity for lactobacillus against pathogen and role of S-layer proteins in adhesion.

2. Materials and methods

2.1. Separation and pure culture of lactobacilli from the intestinal tract of a piglet

Intestinal chyme and mucus were collected from a healthy weaned piglet at age of 30 d. After homogenizing, they were spread on de Man, Rogosa (MRS) agar (Hangzhou microbial reagent CO., LTD) for selective culture of lactobacilli. Agar plates were incubated anaerobically at 37 °C for 2 days, then visible colonies were identified using microscope after being stained by methylene blue. Selected colonies were cultured anaerobically on agar plates for three times for purification. The purified lactobacilli were cultured in de Man, Rogosa (MRS) broth anaerobically at 37 °C for 2 days and stored at –80 °C in MRS broth supplemented with 20% (v/v) glyceral until use.

2.2. 16S rRNA sequencing and phylogenetic analysis

DNA was extracted from lactobacilli with the Gram-positive bacteria DNA Extract Kit (Hangzhou Xinjie biotechnology company Ltd.), and full-length 16S rRNA gene fragments were amplified by PCR with primers 10F and 1500R [24]. The PCR products were sequenced and deposited in Genbank for Blast analysis (http://blast.ncbi.nlm.nih.gov/BLAST/) alignment with Clustal W. The phylogenetic analysis of lactobacillus sequences was conducted with MEGA 3.0.

2.3. Caco-2 cell culture and adhesion assay of lactobacillus

The Caco-2 cell line was purchased from the Chinese Academy of Science Collection Center (Institute of Biochemistry and Cell Biology, Shanghai, China). The cells were cultured in minimal essential medium (MEM) (Gibico, USA) supplemented with 10% (v/v) fetal bovine serum (Gibico, USA), 100 U/ml penicillin and 100 U/ml streptomycin (Sijiqing Co. Ltd., Zhejiang, China) in an incubator at 37 °C in the presence of 5% CO2 (v/v). The cell culture medium was changed every two days. The Caco-2 cells with 80–90% confluence were used.

Depending on the phylogenetic analysis and the shape of the colonies under microscope stained by methylene blue, forty strains were chosen to adhere to Caco-2 cells. All lactobacillus strains were grown in MRS broth at 37 °C for 20 h, the optical density was adjusted to 3 × 10⁸ cfu/ml with PBS (pH 7.4) according to the method describe previously [25,26]. The ability of adhesion of lactobacillus to caco-2 cells was assessed by the method used as previously with some modifications [27]. The Caco-2 cells were cultured in six-well plates and washed three times with phosphate-buffered saline (PBS, pH 7.4) before use. One ml of lactobacillus in PBS (10⁵ cfu/ml) and 1 ml MEM medium with 10% (v/v) fetal bovine serum (Gibico, USA) were added to each well, then the plates were incubated at 37 °C with 5% CO2 (v/v). After 2 h of incubation, the monolayer cells were washed three times with PBS, fixed with 10% (v/v) methanol for 15 min, Gram-stained, and then examined by microscope. The adherence of lactobacillus index was determined as per 100 Caco-2 cells in 20 random microscopic fields. Adhesion experiments were performed in triplicate.

2.4. Characterization of heat, acid, bile salt and metal (Zn²⁺ and Cu²⁺) tolerance

The five selected strains with high adhesive ability to Caco-2 cells and commercial strain LGG were evaluated for the tolerance to heat, acid, bile salt and metal (Zn²⁺ and Cu²⁺). The strains cultured in MRS broth were treated under 55 °C for 20 min for the heat tolerance evaluation. The strains were cultured in the MRS broths with pH 2.0 or 0.1% bile salt (Hangzhou microbial reagent CO., LTD) for 4 h for the acid or bile salt tolerance determination, respectively. The number of viable lactobacillus strains was determined by plating on MRS agar plates after serial dilutions for heat, acid and bile salt tolerance, respectively. For the metal (Zn²⁺ and Cu²⁺) tolerance assays, the strains were cultured in liquid MRS broths with CuSO4 (50 mg/L Cu²⁺) or ZnSO4 (600 mg/L Zn²⁺). The strains stressed under metal compound were incubated for 24 h and the absorbance of culture liquid was detected at 600 nm.

2.5. Isolation and SDS-PAGE analysis of S-layer proteins from lactobacillus

S-layer proteins of lactobacillus were extracted by 5 M LiCl according to the method reported before with some modification [27]. Six selected lactobacilli with high or low adhesive ability and LGG were incubated in 30 ml MRS broth, respectively. After cultured for 18 h, cells were collected and washed twice with ice-cold sterile water. Six ml of 5 M LiCl was used to mix with lactobacilli. Supernatant was collected and dialyzed with PBS, then freeze drying. SDS-PAGE was performed with a 5% (w/v) stacking gel and a 12% (w/v) separating gel. Sixteen μl of protein solution was resuspended in 4 μl loading buffer, boiled for 5 min, centrifuged, and loaded on gel. Gels were stained by Coomassie brilliant blue R-250 (Sigma).

2.6. Inhibition of E. coli k88 and Salmonella enteritidis 50335 adherence to Caco-2 cells by lactobacillus

The inhibition of Lactobacillus to pathogens adherence was performed according to the previous method with some modification [28]. Seven lactobacilli as mentioned above were used. The optical density was adjusted to 3 × 10⁸ cfu/ml with PBS (pH 7.4). Three different procedures, competition, exclusion and displacement, were used to evaluate the inhibition ability of lactobacillus with or without S-layer protein to pathogen adherence to Caco-2.
E. coli k88 was from National Center for Veterinary Culture Collection (CVCC, China) and S. enteritidis 50335 was from National Center for Medical Culture Collection (CMCC(B), China), respectively.

For competition assays, 200 μl (approximately $1 \times 10^6$ cfu) lactobacillus and 200 μl (approximately $1 \times 10^6$ cfu) pathogens were co-cultured with Caco-2 cells in MEM for 2 h. For exclusion assays, lactobacillus was cultured with Caco-2 cells in MEM for 1 h. After Caco-2 cells were washed three times with PBS (pH 7.4), pathogens were added for further incubation for 1 h. For displacement assays, pathogens were added and cultured for 1 h, and then the lactobacillus were added and cultured for 1 h. After culture, the cells were lysed by addition of 0.05% (v/v) Triton-X100 solutions on ice for 10 min and the number of viable adhering E. coli k88 and S. enteritidis 50335 were determined by plating on EMB and SS agar plates after serial dilutions, respectively. The inhibition of pathogens by lactobacillus without S-layer protein was also conducted as above.

3. Results

3.1. 16S rRNA gene phylogeny of lactobacillus

Eighty-five lactobacillus strains were obtained, including one Lactobacillus johnsonii, 54 L. salivarius and 30 L. reuteri strains (Fig. 1). Identity of 16S rRNA sequence to the published sequences in NCBI was larger than 99% in all cases.

3.2. Selection of lactobacillus through adhesion capacity

Five lactobacilli from 40 strains including 4 L. reuteri and 1 L. salivarius strain were found to have higher adhesive ability (Table 1). The number of adhesion for five lactobacilli strains ranged from 4.9 to 15.9 cfu/Caco-2 cell, and were significantly higher than others ($p < 0.05$). Two typical strains with higher (ZJ617) and lower adhesive ability (ZJ610) adhering to Caco-2 cells are shown in Fig. 2.

3.3. Tolerance ability of five selected lactobacilli to heat, acid, bile and metal

The survival rate of lactobacillus strains ZJ614, ZJ616, ZJ617, ZJ621 and ZJ623 treated with heat or Zn$^{2+}$ was significantly lower than that of LGG ($P < 0.05$, Fig. 3). Under acid condition, strain ZJ621 had significantly higher survival rate than LGG ($P < 0.05$), while the others were lower than LGG. The survival rate of lactobacillus strains ZJ616, ZJ617, ZJ621 and ZJ623 cultured in medium with Cu$^{2+}$ was significantly higher than that of LGG ($P < 0.05$).

3.4. SDS-PAGA analysis of S-layer proteins

Seven strains had different pattern of bands ranging from 34 to 130 kDa including the major molecular masses approximately 42 KD, as shown in Fig. 4. Six lactobacilli isolated from intestinal tract of piglet had richer S-layer proteins compared with LGG. The
abundance of proteins from ZJ610 with lower adhesion ability (0.12 \( \pm \) 0.02 cfu/cell) was lower than those of ZJ616, ZJ617, ZJ621, ZJ614 and ZJ623 with higher adhesion capacity.

3.5. The inhibition of lactobacillus to E. coli k88 and S. enteritidis 50335 and the role of S-layer protein in function

Inhibition of E. coli k88 and S. enteritidis 50335 adherence to Caco-2 cells by Lactobacillus with or without S-layer protein is shown in Tables 2 and 3, respectively. All the lactobacillus strains significantly inhibited the adhesion of E. coli k88 and S. enteritidis 50335 to Caco-2 cells (\( P < 0.05 \)).

In competition assay, the inhibition activity of strains ZJ614, ZJ616, ZJ617, ZJ621, ZJ623 and LGG against E. coli k88 and S. enteritidis 50335 was much higher than that of lactobacillus ZJ610 (\( P < 0.05 \)), with an except of ZJ617 to E. coli k88. The selected lactobacillus ZJ614, ZJ616, ZJ617, ZJ621, ZJ623 inhibited 18.8, 32.9, 15.7, 29.1 and 19.2% of the adherence of E. coli k88 to Caco-2 cells, respectively, while lactobacillus ZJ623 showed the highest inhibition ability against S. enteritidis 50335 up to 34.5%. After the S-layer proteins were removed by 5 M LiCl, the inhibition activity of ZJ614, ZJ616, ZJ621, and ZJ623 against S. enteritidis 50335 were significantly reduced.

Table 1

<table>
<thead>
<tr>
<th>Strain</th>
<th>Species</th>
<th>Accession number</th>
<th>CFU/cell</th>
</tr>
</thead>
<tbody>
<tr>
<td>ZJ602</td>
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<td>JN081844</td>
<td>2.26 ± 0.32e</td>
</tr>
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<td>ZJ604</td>
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<td>JN081846</td>
<td>1.62 ± 0.35f</td>
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<td>ZJ613</td>
<td>Lactobacillus salivarius</td>
<td>JN081855</td>
<td>2.18 ± 0.20c</td>
</tr>
<tr>
<td>ZJ614</td>
<td>Lactobacillus salivarius</td>
<td>JN081856</td>
<td>4.90 ± 0.08d</td>
</tr>
<tr>
<td>ZJ615</td>
<td>Lactobacillus reuteri</td>
<td>JN081857</td>
<td>1.21 ± 0.14</td>
</tr>
<tr>
<td>ZJ616</td>
<td>Lactobacillus reuteri</td>
<td>JN081858</td>
<td>15.87 ± 0.31a</td>
</tr>
<tr>
<td>ZJ617</td>
<td>Lactobacillus reuteri</td>
<td>JN081859</td>
<td>12.35 ± 0.09b</td>
</tr>
<tr>
<td>ZJ618</td>
<td>Lactobacillus reuteri</td>
<td>JN081860</td>
<td>4.68 ± 0.10c</td>
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<td>ZJ621</td>
<td>Lactobacillus reuteri</td>
<td>JN081863</td>
<td>8.51 ± 0.09f</td>
</tr>
<tr>
<td>ZJ622</td>
<td>Lactobacillus reuteri</td>
<td>JN081864</td>
<td>1.01 ± 0.20f</td>
</tr>
<tr>
<td>ZJ623</td>
<td>Lactobacillus johnsonii</td>
<td>JN081865</td>
<td>7.91 ± 0.16d</td>
</tr>
<tr>
<td>ZJ626</td>
<td>Lactobacillus salivarius</td>
<td>JN081868</td>
<td>2.45 ± 0.10f</td>
</tr>
<tr>
<td>ZJ610</td>
<td>Lactobacillus salivarius</td>
<td>JN081852</td>
<td>0.12 ± 0.02b</td>
</tr>
<tr>
<td>Other</td>
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<td>—</td>
<td>&lt;1.00</td>
</tr>
<tr>
<td>SEM</td>
<td>—</td>
<td>0.081</td>
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</tr>
</tbody>
</table>

Data are mean ± standard deviation. Different symbol means statistically significant difference (\( p < 0.05 \)). Other means the other 27 strains with adhesive ability lower than 1.00 cfu/cell, which are not showed in Table 1.

Other means the other 27 strains with adhesive ability lower than 1.00 cfu/cell, which are not showed in Table 1.

Fig. 2. Typical lactobacillus strains with high and low adhesive ability. (A) Lactobacillus ZJ617 with high adhesive ability (12.35 ± 0.09 cfu/cell), (B) Lactobacillus ZJ610 with low adhesive ability (0.12 ± 0.02 cfu/cell). The adhesive strains were Gram-stained and examined with light microscope at 100x oil immersion lens.

Fig. 3. The heat, pH, bile (A) and metal (Zn and Cu) (B) tolerance ability of Lactobacillus strain. The survival number of lactobacillus strains was detected after treated by heat at 55°C for 20 min, PH 2.0 or Bile 0.1% for 4 h. The OD600 value of liquid culture was evaluated after lactobacillus strains treated by 50 mg/L CuSO4 or 600 mg/L ZnSO4 for 24 h. Different symbol means statistically significant difference (\( p < 0.05 \)) within the same treatment.
coli k88 adhering to the cells, higher than ZJ614 (22.2%), ZJ616 S. enteritidis 50335 with the same to the strains ZJ616, ZJ621, ZJ623 and LGG against ability. Without S-layer proteins, the inhibition activity of ZJ614, and ZJ623, respectively, with ZJ623 showing the highest inhibitive 28.3, 17.6, 32 and 34.4% were inhibited by ZJ614, ZJ616, ZJ617, ZJ621 S. enteritidis 50335 except LGG showed signi

Fig. 4. SDS-PAGE analysis of S-layer proteins extracted with 5 M LiCl from Lactobacillus ZJ610, ZJ616, ZJ617, ZJ621, ZJ614, ZJ623 and LGG. M: low molecular weight protein standards. Seven strains had different bands ranging from 34 to 130 kDa including the major molecular masses approximately 42 kD. The abundance of S-layer proteins from lactobacillus ZJ616, ZJ617, ZJ621, ZJ614 and ZJ623 with higher adhesion capacity (4.90–18.57 cfu/cell) was higher than that of ZJ610 with lower adhesion ability (0.12 ± 0.02 cfu/cell).

(p < 0.05), but those of the other three strains were not. With S-layer protein removed, ZJ621 and LGG inhibition activity against E. coli k88 significantly reduced (p < 0.05).

In exclusion assay, the strains ZJ616, ZJ17, ZJ621 and LGG have higher inhibition ability against E. coli k88 than ZJU 610 (p < 0.05), with the same to the strains ZJ616, ZJ621, ZJ623 and LGG against S. enteritidis 50335 than ZJ610. Lactobacillus ZJ621 inhibited 33% of E. coli k88 adhering to the cells, higher than ZJ614 (22.2%), ZJ616 (25.7%), ZJ617 (25.3%), ZJ623 (21.5%). For S. enteritidis 50335, 22,1, 28,3, 17,6, 32 and 34.4% were inhibited by ZJ614, ZJ616, ZJ617, ZJ621 and ZJ623, respectively, with ZJ623 showing the highest inhibitive. Without S-layer proteins, the inhibition activity of ZJ614, ZJ616, ZJ621, ZJ623 and LGG were significantly reduced (p < 0.05) against S. enteritidis 50335, and the inhibition activity of ZJ614, ZJ621 and LGG were also significantly reduced against E. coli k88 (p < 0.05).

In displacement assay, all the strains with high adhesion activity except LGG showed significantly higher inhibition ability against S. enteritidis 50335 than ZJ610 (p < 0.05), among which, ZJ614 and ZJ623 decreased by 24% and 25.1% of S. enteritidis 50335 to adhere to Caco-2, respectively. The inhibition ability of ZJ614, ZJ617, ZJ621, ZJ623 and LGG against E. coli k88 was significantly higher than that of ZJ610, and ZJ621 had the highest inhibition ability to decrease 24.9% of the E. coli k88. When S-layer proteins removed, the inhibition activity of strains ZJ621, ZJ623 and LGG against S. enteritidis was significantly reduced (p < 0.05), the same with ZJ614, ZJ621 and LGG against E. coli k88 (p < 0.05).

4. Discussion

Many lactobacillus strains used as probiotics instead of antibiotic showed beneficial effect on animal health. However, the mechanism of lactobacillus to adhere to the intestine tracts and inhibit pathogens remains unclear. Adhesion of lactobacillus strains to the intestinal cells was considered to be one of the most crucial criteria to select probiotic strains [28,29]. The strains with high adhesion ability can efficiently occupy the adhesive sites on the intestinal cells and mucous to inhibit the adhesion of pathogens and protect the host cells. Five strains isolated for adhesion capacity from the intestine of a piglet in this study had much higher adhesion ability that Lactobacillus paracasei M5-L, Lactobacillus rhamnosus J10-L and Lactobacillus casei Q8-L [27] and L. reuteri JCMI0181 [30], indicating that the ability of adhesion varies with the lactobacillus species, even the same lactobacillus genus. Many lactobacillus strains can inhibit the adhesion of pathogens to the intestinal epithelial cell and mucous. The inhibition of adhesion of different pathogens was specifically depending on the strains and pathogens used as well as the methods of assessment [15,31]. The commercial strain LGG showed higher inhibition efficiency against E. coli k88 than S. enteritidis 50335. Five strains isolated with high adhesion ability did not show the same inhibition capacity against E. coli k88 and S. enteritidis 50335, but they efficiently inhibited the adhesion of both pathogenic bacteria to Caco-2 cell in all three assays. It is reported that L. casei rhamnosus 35 can interfere with the adhesion of enterotoxicogen and enteropathogenic E. coli [28]. The five strains with high adhesion ability generally showed much higher inhibition ability to the adherence of pathogen to the intestine epithelial cells than the representative strain ZJ610 with a low adhesive ability, indicating that the inhibition capacity of lactobacillus against pathogenic bacteria may be related to the adhesion ability. However, the inhibition ability of ZJ621 against E. coli k88 was significantly higher than the other four strains in competition and displacement assays, while the adhesion ability of ZJ621 was not the highest one among the five strains. Meanwhile, the inhibition ability against the E. coli k88 and S. enteritidis 50335 by the five strains with high adhesive ability did not increase as the adhesive ability of the strains increased. Collado et al. [9] found that some commercial strains with low adhesive activity had better inhibition ability compared with other high adhesive strains. Higher adhesion ability is not always associated with higher inhibition capacity against pathogens, suggesting that the inhibition capacity is complicated and many factors may be involved.

Table 2

<table>
<thead>
<tr>
<th>Strain</th>
<th>Competition L + S</th>
<th>L - S</th>
<th>Exclusion L + S</th>
<th>L - S</th>
<th>Displacement L + S</th>
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<td>ZJ610</td>
<td>89.5 ± 3.6A</td>
<td>88.2 ± 5.8B</td>
<td>80.9 ± 2.4A</td>
<td>81.3 ± 12.8A</td>
<td>87.8 ± 3.8A</td>
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<td>ZJ616</td>
<td>67.1 ± 5.9f</td>
<td>73.6 ± 6.9f</td>
<td>74.3 ± 1.4f</td>
<td>78.7 ± 11.8f</td>
<td>81.1 ± 5.2f</td>
<td>82.4 ± 6.6f</td>
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<td>ZJ617</td>
<td>84.3 ± 5.9m</td>
<td>91.0 ± 5.8m</td>
<td>74.7 ± 2.4m</td>
<td>80 ± 11.3m</td>
<td>80.1 ± 6.4m</td>
<td>83.8 ± 6.4m</td>
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<td>ZJ621</td>
<td>70.9 ± 8.1cx</td>
<td>81.9 ± 5.9Rv</td>
<td>67.0 ± 2.8cx</td>
<td>80.3 ± 11.3Y</td>
<td>75.1 ± 8.6x</td>
<td>92.0 ± 6.3y</td>
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<tr>
<td>ZJ614</td>
<td>81.2 ± 3.13h</td>
<td>81.6 ± 5.8Rc</td>
<td>77.8 ± 2.8xrc</td>
<td>90.7 ± 6.5Xv</td>
<td>76.6 ± 5.0Rxx</td>
<td>93.0 ± 4.1y</td>
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<tr>
<td>ZJ623</td>
<td>80.8 ± 5.6e</td>
<td>84.4 ± 5.5ne</td>
<td>78.5 ± 3.7e</td>
<td>82.7 ± 13.1A</td>
<td>77.8 ± 4.1f</td>
<td>79.6 ± 2.9f</td>
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<td>LGG</td>
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<td>81.1 ± 9.7Ryc</td>
<td>65.1 ± 3.75x</td>
<td>78.7 ± 11.8Xv</td>
<td>67.8 ± 5.8ecx</td>
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Data are adherence ratio of E. coli k88 to Caco-2 cells = (test/control) × 100%, shown as mean ± standard deviation of three independent experiments. L + S means lactobacillus with S-layer proteins, L - S means lactobacillus without S-layer proteins after treatment by 5 M LiCl.

A-BDifferent symbol means statistically significant difference (p < 0.05) within the same row.

XDifferent symbol means statistically significant difference (P < 0.05) within the same row between the treatments L + S and L - S.
S-layer proteins were identified in many lactobacilli. In a previous study, 8% of the strains from the 99 isolates tested were found to produce S-layer proteins [19]. The SDS-PAGE showed that the strains except ZJ610 with low adhesivity isolated had abundant S-layer proteins and these strains had the bands ranging from 34 to 130 kDa, which is in agreement with the results by Ref. [15,30], reported that L. reuteri JCM1081 had S-layer proteins ranging from 30 to 130 kDa, and Lactobacillus acidophilus L050103-12, L. rhamnosus 1.120 and Lactobacottus bulgaricus N had several S-layer protein bands. However, many strains only have one or two bands [4,32]. The result may vary with the lactobacillus strains used and the methods used for extraction of the S-layer proteins.

For some lactobacillus strains, S-layer proteins perform as adhesion medium binding lactobacillus to the intestinal epithelial cells and mucus, such as mucus-binding proteins MapA from L. reuteri [33] and surface-protein from Lactobacottus planuntarum 423 [34]. Moreover, adhesive properties of S-layers to matrix components have been linked to protective functions against invasiveness of pathogenic bacteria and to the probiotic properties of benefiting bacteria [18]. S-layer proteins of several lactobacilli, including L. crispatus and L. acidophilus whose ability to bind to host epithelial cells is decreased after removal or disruption of the S-layer proteins [23,35,36], have been shown to confer tissue adherence. After the lactobacilli pretreated with protease or LiCl to remove S-layer protein, the inhibition ability of lactobacillus against pathogens decreased [41,30,37]. In this study, the inhibition capacity of the isolates ZJ614, ZJ616, ZJ621, ZJ623 and LGG against S. enteritidis 50335 was reduced when they were treated with 5 M LiCl. The removal of S-layer proteins from ZJ621 and LGG significantly affected the inhibition ability against E. coli K88 (p < 0.05). The role of S layer proteins may differ in the inhibition against pathogens between E. coli K88 and S. enteritidis 50335. Blotting assays performed with S. enteritidis pretreated with S-layer proteins from Lactobacillus kefir strains clearly showed that those proteins remained associated with Salmonella surface and could either modify or mask Salmonella structures necessary for the invasion of cultured human enterocytes, which demonstrated that when S. enteritidis is preincubated with L. kefir S-layer there is a direct interaction between this protein and S. enteritidis surface, instead of a competition for binding sites on the surface of the enterocyte [32]. S-layer from Lactobacillus in this study may blot with surface molecular on S. enteritidis and function in inhibition. On the other hand, although E. coli K88 and S. enteritidis 50335 are both Gram-negative bacteria with adhesive fimbriae, three expressed K88 fimbrial adhesin variants of E. coli K88 have been proved to be lectins which are specifically recognized by carbohydrate structures expressed on host cell or mucus [38–41]. For S. enteritidis, both fimbrial and non-fimbrial adhesins are the two major groups of adhesive structures targeting more receptors than carbohydrate structures on host [42]. It is deemed that S-layer protein may function with not only carbohydrate structures but also molecular on host cell to inhibit the adhesion of pathogenic bacteria. The adhesins of lactobacillus strains, E. coli and S. enteritidis to host have been well studied, but the receptors expressed on host involved in adhesion are still unclear. Further researches are needed to explain the adhesion mechanism. Removal of S-layer protein did not affect the inhibition capacity of ZJ617 against S. enteritidis 50335 and E. coli K88. Thus, it is inferred that surface structures other than the S-layer protein mediate adhesion in these strains. For example lipoteichoic acid has been confirmed to participate in the adhesion process [19]. The adhesion of Lactobacillus brevis ATCC 8287 and some porcine Lactobacillus isolates to fibronectin and laminin was reduced after the removal of S-layer proteins, whereas some of the isolates possessed better adhesiveness to laminin after abolishment of the S-layer structure [19].

In conclusion, the five strains with high adhesive ability compared with others showed great inhibition capacity against E. coli K88 and S. enteritidis 50335 to adhere to intestinal epithelial cells. The results indicate that higher adhesive ability means higher inhibition activity for lactobacillus against pathogen, in which S-layer proteins plays an important role.

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