Inhibition of *Shigella sonnei*-induced epithelial barrier disruption by surface-layer associated proteins of lactobacilli from Chinese fermented food

Yingchun Zhang,*¹ Xiaolu Shi,* Siqi Hao,* Qianhui Lu,* Lanwei Zhang,† Xue Han,* and Weihong Lu*

*Department of Food Science and Engineering, School of Chemistry and Chemical Engineering, Harbin Institute of Technology, Harbin 150001, China

†College of Food Science and Engineering, Ocean University of China, Qingdao 266003, China

ABSTRACT

Surface-layer associated proteins (SLAP) of *Lactobacillus paracasei* ssp. *paracasei* M5-L and *Lactobacillus casei* Q8-L were examined to identify the functional basis for their protection within intestinal epithelial cells. The results showed that SLAP of M5-L and Q8-L remained active in a trypsin solution and retained a 45-kDa protein band, similar to that observed in controls. In contrast, under conditions of simulated gastric juice, the SLAP were partially degraded. Inhibitory effects of SLAP on adherence of *Shigella sonnei* to HT-29 cells were assessed with use of exclusion, competition, and replacement assays. In response to M5-L at 50 µg/mL SLAP, an inhibition ratio of 33% was obtained, while for Q8-L at 400 µg/mL SLAP, the inhibition ratio was 48%. Hoechst 33258 test results showed that cells infected with *S. sonnei* and co-incubated with SLAP of M5-L and Q8-L were only partially apoptotic, with apoptosis rates of 37.67 and 43.67%, respectively. These levels of apoptosis were substantially lower than that observed with cells infected with *S. sonnei* alone. In addition, the SLAP of Q8-L and M5-L reduced downstream caspase-1 activity and further modified apoptotic cell damage. Finally, SLAP of M5-L and Q8-L were also able to prevent *S. sonnei*-induced membrane damage by inhibiting delocalization of zonula occludens (ZO)-1 and reducing the amount of occludin produced by *S. sonnei*.

Key words: surface-layer associated proteins, lactobacilli, inhibition, *Shigella sonnei*

INTRODUCTION

Shigella, a pathovar of *Escherichia coli*, is composed of 4 distinct species: *Shigella flexneri*, *Shigella sonnei*, *Shigella dysenteriae*, and *Shigella boydii*. *Shigella* accounts for the majority of dysentery invading the human gut

mucosa and is the basis for most gastrointestinal illness infections occurring worldwide (Anderson et al., 2016). Direct contact with and invasion of host epithelial and immune cells are the main modes of host–pathogen interactions, a process that is particularly characteristic of *Shigella* (Tran Van Nhieu et al., 2000). Pathogenic bacteria can regulate tight junction (TJ) structures of epithelial cells, and the role of TJ in infection has been widely demonstrated. Specifically, disruption of TJ leads to a distinct increase in paracellular permeability and polarity defects, which then facilitate viral or bacterial entry and subsequent dispersal. In addition to pathological changes in TJ integrity, TJ proteins such as occludin and claudins can either function as receptors for pathogen entry or interact with viral or bacterial effector molecules, which represents an essential step for infection (Lu et al., 2014).

Many lactobacilli have been characterized as probiotics because they possess the ability to exert health benefits for the host. The activities of probiotics have been investigated and several modes of action proposed, including co-aggregation with pathogens, competitive exclusion, contribution to mucosal barrier function, antimicrobial activity through reductions in luminal pH, secretion of specific compounds such as bacteriocins, and the modulation of immune responses (Golowcyc et al., 2007; Breuer, 2009; Šušković et al., 2010; Bron et al., 2011). Adhesion to intestinal epithelial cells is considered the first step for the probiotic function of lactobacilli. Lactobacilli with high adhesive abilities have a greater gut residence time, increase the exclusion of pathogens, and protect epithelial cells (Servin, 2004; Granato et al., 1999). Accordingly, the survival and persistence of lactobacilli within the gastrointestinal tract represents an important factor with regard to their beneficial effects (Zhang et al., 2013).

Many *Lactobacillus* species possess surface-layer associated proteins (SLAP), which are surface structures consisting of 2-dimensional arrays of proteinaceous subunits that are found among the most commonly observed prokaryotic cells (Sleytr, 1976; Sleytr et al., 2014). Several functions of SLAP have been reported,

Received June 29, 2017.

Accepted November 22, 2017.

¹Corresponding author: zyc229@163.com

such as protection against hostile environments, maintenance of cellular shape, control of nutrient and metabolite transfer, and they play an important role in the colonization of *Lactobacillus acidophilus* within the gastrointestinal tract (GIT; Beveridge et al., 1997; Chen et al., 2007). The SLAP are also involved in mediating adhesion of lactobacilli cells to intestinal epithelium cells (Khang et al., 2009; Sun et al., 2013), as well as inhibiting *Salmonella*-induced reorganization of the cytoskeleton (Li et al., 2012). The adhesive quality of intestinal epithelia allows lactobacilli to prolong their probiotic effects (Avall-jääskeläinen et al., 2003; Jakava-Viljanen and Palva, 2007).

The isolates *Lactobacillus paracasei* ssp. *paracasei* M5-L and *Lactobacillus casei* Q8-L, which can survive passage through the GIT and exert antimicrobial activity against 3 pathogens (*Shigella sonnei* ATCC 25931, *Escherichia coli* ATCC25922, and *Salmonella enterica* serovar Typhimurium ATCC 14028), have been demonstrated to adhere effectively to HT-29 cells (Zhang et al., 2011). In addition, M5-L and Q8-L possess the ability to inhibit adherence of *S. sonnei* to HT-29 cells (Zhang et al., 2010). The SLAP of M5-L and Q8-L have been identified as surface antigens and the elongation factor Tu (GI number: 489671527), respectively, as determined by liquid chromatography-tandem MS (Zhang et al., 2016).

The main objective of this study was to evaluate the inhibitory effects of SLAP on adherence of *S. sonnei* to HT-29 cells by using exclusion, competition, and replacement assays. In addition, we evaluated the protective ability of SLAP on intestinal epithelial cells to verify that M5-L and Q8-L could serve as novel probiotic strains for use in the food industry. The results of this study could help to identify probiotic candidates for further investigation in vivo.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions

Lactobacillus paracasei ssp. *paracasei* M5-L was isolated from kumiss produced in local households in Sinkiang (China), whereas *L. casei* Q8-L was isolated from yak yogurt produced in local households in Qinghai, China (Zhang et al., 2011). Both strains were stored in our laboratory and cultured in de Man, Rogosa, and Sharpe (Difco/Becton Dickinson Co., Franklin Lakes, NJ) broth under aerobic condition at 37°C. The indicator bacteria used for antimicrobial assays were cultured in tryptone soy agar (TSA) or broth in aerobic conditions at 37°C and included *S. sonnei* ATCC 25931 provided by the Microbiological Laboratory of the Clinical Detection Center of Heilongjiang (Harbin, China).

Extraction of SLAP

The SLAP of *L. paracasei* ssp. *paracasei* M5-L and *L. casei* Q8-L were extracted with 5 M LiCl as described previously (Lortal et al., 1992; Smit et al., 2001). Briefly, cells obtained from 500 mL of de Man, Rogosa, and Sharpe broth after 16 h of incubation were collected by centrifugation ($8,000 \times g$ at 10°C for 10 min), washed twice with ice-cold sterile distilled water (overnight at 4°C), and then mixed with 50 mL of 5 M LiCl. The mixture was incubated in a shaking incubator (SPX-150C, Boxun, China) at 150 rpm and 37°C for 60 min to extract noncovalently bound proteins. The supernatant was then harvested by centrifugation ($10,000 \times g$ at 10°C for 20 min) and dialyzed extensively against distilled water, with the water being changed every 2 h for the first 8 h. Then, the LiCl was removed from the liquid, concentrated with PEG-8000 (polyethylene glycol, Amresco, Solon, OH), and stored at -20°C before being lyophilized.

SLAP of Lactobacilli Activity Under Conditions Simulating the Human GIT

Resistance of the SLAP of M5-L and Q8-L to pepsin under low pH was tested as described previously (Charteris et al., 1998). Briefly, simulated gastric juice was prepared by suspending pepsin (0.3 mg/mL; Sigma, St. Louis, MO) in PBS (pH 2.0). Subsequently, 0.5 mL of SLAP of M5-L or Q8-L were inoculated into 1 mL of this simulated gastric juice and 0.3 mL of NaCl (0.5% wt/vol), mixed, and incubated at 37°C for 1 h. The degradation of SLAP was analyzed using SDS-PAGE. With this protocol, the time spent by food in the stomach could be modeled.

Tolerance of conditions of the small intestine was tested in PBS (pH 8.0) containing trypsin (0.1 mg/mL, Sigma) and 0.3% (wt/vol) Oxgall (Sigma). The SLAP (0.5 mL) of M5-L and Q8-L were inoculated into 1 mL of the simulated small intestine juice, mixed, and incubated at 37°C for 3 h. The degradation of SLAP was analyzed using SDS-PAGE, to test transit tolerance within this simulated small intestine, again providing a model for the time spent by food in the small intestine (Maragkoudakis et al., 2006).

Antimicrobial Activity of SLAP

Cells of *S. sonnei* from 1 mL of TSA culture medium after 12 h of incubation were collected by centrifugation ($8,000 \times g$ at 10°C for 10 min). These cells were then inoculated at varying concentrations with 100 μ L of SLAP (0, 25, 50, or 100 μ g/mL) in RPMI-1640 medium (the cell culture medium was added to the 1 mL of

TSA) and incubated for 2 h at 37°C in 5% CO₂. *Shigella sonnei* cells were then serially diluted and plated on TSA plates to determine their survival. All experiments were independently performed 3 times and each assay was replicated 3 times.

Inhibition by SLAP of *S. sonnei* Adherence to HT-29 Cells

The ability for SLAP to inhibit *S. sonnei* adherence to HT-29 cells was assayed according to methods reported previously with some modifications (Candela et al., 2008; Ramiah et al., 2008). In brief, approximately 3 to 4 × 10⁵ cells per well were seeded in a 12-well plate (Corning Inc., Corning, NY). The culture medium was changed daily and HT-29 monolayers at 80 to 90% confluence were washed twice with PBS (pH 7.4) before experiments.

In the exclusion assay, HT-29 monolayers were inoculated with 800 µL of RPMI-1640 medium; then, varying concentrations of 100 µL of SLAP (25, 50, 100, or 400 µg/mL) were added to the HT-29 cells and incubated for 60 min at 37°C in 5% CO₂, followed by atmospheric incubation for 1 h at 37°C in 5% CO₂. The HT-29 monolayers were then inoculated with 100 µL of *S. sonnei* suspension (10⁷ cfu/well) in RPMI-1640 medium and incubated for 1 h at 37°C in 5% CO₂.

In the competition assay, HT-29 monolayers were inoculated with 800 µL of RPMI-1640 medium, and a mixture of varying concentrations of 100 µL of SLAP (25, 50, 100, or 400 µg/mL) and 100 µL of *S. sonnei* suspension (10⁷ cfu/well) in RPMI-1640 medium were added into each well and cultured for 2 h at 37°C.

In the displacement assay, HT-29 monolayers were inoculated with 800 µL of RPMI-1640 medium and 100 µL of *S. sonnei* suspension (10⁷ cfu/well) in RPMI-1640 medium and incubated for 1 h at 37°C in 5% CO₂. Nonadhering cells were removed by washing twice with sterile PBS. The HT-29 monolayers were then inoculated with varying concentrations of SLAP (25, 50, 100, or 400 µg/mL) in RPMI-1640 medium and incubated for 1 h at 37°C in 5% CO₂. Wells containing *S. sonnei* alone served as controls.

In all experiments, nonadhering cells were removed by washing 4 times with sterile PBS, and then treated with 0.5 mL of 0.5% (vol/vol) Triton X-100 (Amresco) for 5 min in an ice water bath. The cells were then serially diluted and plated onto TSA plates to count *S. sonnei* cells. The ability of SLAP to exclude, compete with, and displace *S. sonnei* was determined by comparing the adhesion of *S. sonnei* in the presence of SLAP with that of *S. sonnei* alone. The number of *S. sonnei* cells failing to adhere to HT-29 cells was then expressed as

a percentage. All experiments were replicated 3 times and each assay was performed in triplicate.

Effects of SLAP on Apoptosis of Human Cells Using Hoechst 33258 Staining

Cell apoptosis was detected by nucleus staining with Hoechst 33258 as described previously (Allen et al., 2001). Briefly, 2 mL of HT-29 cells (2.5 × 10⁵ cells/well) was cultured in a 6-well plate at 37°C for 6 h after cells had adhered to the wells, followed by addition of 200 µL of SLAP of M5-L (50 µg/mL) or Q8-L (400 µg/mL) and 200 µL of *S. sonnei* (1 × 10⁷ cfu/mL) to the HT-29 cells. Medium (RPMI-1640) was added to the well to achieve a final volume of 2 mL and co-incubated for 12 h at 37°C in 5% CO₂. Treated and control cells were washed twice with PBS and stained using Hoechst 33258 (5 µg/mL from a stock solution of 1 mg/mL in sterile water) for 5 min at 37°C. The stained cells were manually shaken several times. The dye liquor was aspirated and cells were washed twice with PBS and observed immediately under a fluorescence microscope (Olympus/Nikon, Tokyo, Japan) with a filter set (excitation/emission) of BP330–380 nm/LP420 nm. Images were recorded with use of a color charge-coupled device camera.

Caspase-1 Activity Assays

Caspase-1 activity was determined using a kit (Beyotime Institute of Biotechnology, Jiangsu, China), based on the ability of caspase-1 to change acetyl-Tyr-Val-Ala-Asp *p*-nitroanilide (Ac-YVAD-*p*NA) into yellow formazan *p*-nitroaniline. Cell lysates were centrifuged at 12,000 × *g* for 10 min, and the protein concentrations were determined by the Bradford protein assay. Cellular extracts were incubated in a 96-well microtiter plate with 20 ng of caspase substrate acetyl-Asp-Glu-Val-Asp *p*NA (Ac-DEVD-*p*NA) overnight at 37°C. The absorbance values of *p*NA at 405 nm (optical density at 405 nm, OD₄₀₅) were measured using a 96-well plate reader (BioTek, Santa Barbara, CA). An increase in OD₄₀₅ indicated activation of caspase-1 (Singh et al., 2001).

Immunocytochemistry Analysis of Distribution of Zonula Occludens-1 and Occludin

The distribution of zonula occludens-1 (ZO-1) and occludin were analyzed following infection (treatment of HT-29 cells according to the methods described in the subsection Effects of SLAP on Apoptosis of Human Cells Using Hoechst 33258 Staining) using im-

munochemistry and confocal microscopy. Monolayers were washed extensively with PBS and fixed with 2% of paraformaldehyde for 30 min at 37°C. The cells were permeabilized with 0.1% Triton-X100 in PBS for 5 min at 37°C and washed with PBS. Polyclonal rabbit anti-ZO-1 antibody (bs-1329R, Boaoseng, Beijing, China) and polyclonal rabbit anti-occludin antibody (bs-1495R, Boaoseng) were diluted in PBS and incubated with permeabilized cells for 45 min at room temperature in a humid atmosphere. The monolayers were washed and treated with fluorescein-conjugated anti-rabbit IgG (SV-0002, Boaoseng) for 45 min at room temperature in a humid atmosphere. After washing with PBS, the filters were excised from their supports, mounted, and observed under a Leica confocal laser scanning microscope (Leica, Shanghai, China).

Statistical Analysis

All experiments were performed 3 times independently and each assay was performed in duplicate. Results were expressed as means $\pm$ standard deviation. The level of significance was analyzed by ANOVA ($P < 0.05$) using SPSS14.0 for Windows (evaluation version; SPSS Inc., Chicago, IL).

RESULTS

Tolerance of SLAP Under Conditions Simulating the Human GIT

Under conditions of simulating gastric juice, the SLAP of M5-L were degraded to between 35 and 25 kDa (lane 4) compared with controls (lane 2), as shown in Figure 1. Results obtained from SDS-PAGE showed that SLAP of M5-L were degraded to low-molecular-weight proteins. The SLAP of Q8-L were not degraded and the main band of Q8-L SLAP was at 45 kDa (Figure 1; lane 6), which was similar to that of controls (Figure 1; lane 7). These findings indicate that the SLAP of Q8-L demonstrated strong tolerance to degradation after incubation with simulated gastric juice for 1 h (Figure 1). No protein band associated with pepsin was displayed as shown in Figure 1.

In contrast to results obtained under conditions of simulated gastric juice, the SLAP of M5-L and Q8-L remained active in trypsin solution at pH 8.0 and in the presence of bile salts (Oxgall, 0.3% wt/vol). Even after 4 h of exposure, they retained 45-kDa protein bands, as shown in Figure 1 (lanes 3 and 5, respectively), which was similar to that observed in controls (lanes 2 and 7, respectively). No protein band associated with trypsin was displayed as shown in Figure 1 (lane 8).

Antimicrobial Activity of SLAP

Neither SLAP of M5-L nor Q8-L showed any significant antimicrobial activity compared with the control group ($P > 0.05$). The viability of *S. sonnei* did not decrease and different concentrations of SLAP did not affect the survival of *S. sonnei* (Table 1). Based upon these results, we can exclude the possibility that any antimicrobial activity of the tested SLAP was exerted upon *S. sonnei*.

SLAP Inhibition of *S. sonnei* Adherence to HT-29 Cells

At different concentrations, SLAP of M5-L showed varying effects on *S. sonnei* adherence to HT-29 cells in competition, exclusion, and displacement assays, as summarized in Figure 2A. The SLAP of M5-L were more effective in their capacity to inhibit *S. sonnei* adherence to HT-29 cells in competition and exclusion assays than in the displacement assay. Overall, 50 μ g/mL SLAP showed maximal inhibition rates on *S. sonnei* with adherence prevented at 45.49, 48.12, and 33.08% [% *S. sonnei* prevented from adherence to HT-29 cells / (control – test)/control] in competition, exclusion, and displacement assays, respectively ($P < 0.05$). Data are means $\pm$ standard deviation of 3 independent experiments; *S. sonnei* alone served as a control.

Figure 1. Sodium dodecyl sulfate-PAGE of surface-layer proteins under in vitro conditions simulating gastric and intestinal juice. M = low-molecular-weight protein standards; lane 1 = trypsin alone; lane 2 = surface-layer associated protein (SLAP) of *Lactobacillus paracasei* M5-L; lane 3 = SLAP of M5-L treated with trypsin; lane 4 = SLAP of M5-L treated with pepsin; lane 5 = SLAP of *Lactobacillus casei* Q8-L treated with trypsin; lane 6 = SLAP of Q8-L treated with pepsin (lane 6); lane 7 = SLAP of Q8-L; and lane 8 = pepsin alone.

Table 1. Antimicrobial activity of surface-layer associated proteins (SLAP) of *Lactobacillus paracasei* ssp. *paracasei* M5-L and *Lactobacillus casei* Q8-L on *Shigella sonnei* (0 $\mu\text{g/mL}$ SLAP of M5-L or Q8-L was the baseline level for *S. sonnei*)¹

SLAP	<i>Shigella sonnei</i> ($\times 10^9$ cfu/mL)
SLAP of M5-L ($\mu\text{g/mL}$)	
0	1.93 ± 0.10^a
25	2.28 ± 0.16^a
50	2.05 ± 0.04^a
100	2.00 ± 0.04^a
SLAP of Q8-L ($\mu\text{g/mL}$)	
0	1.84 ± 0.06^a
25	1.85 ± 0.17^a
50	1.99 ± 0.06^a
100	1.82 ± 0.16^a

^aMeans with the same superscript letter do not differ significantly ($P < 0.05$).

¹Data are means $\pm$ SD of 3 independent experiments; 0 $\mu\text{g/mL}$ of SLAP alone served as a control.

The most effective inhibition rates in response to varying concentrations of SLAP of Q8-L on *S. sonnei* were obtained in the exclusion assay. The SLAP of Q8-L at 400 $\mu\text{g/mL}$ significantly ($P < 0.05$) inhibited *S. sonnei* and adherence of *S. sonnei* to HT-29 cells was inhibited 37.84, 40.60, and 40.60% in the exclusion, competition, and displacement assays, respectively ($P < 0.05$; Figure 2B).

Effects of SLAP on *S. sonnei*-Induced Human Cell Apoptosis

Under fluorescence microscopy, normal cells showed a uniform dispersion of circular fluorescence and displayed many villus projections on their surface and an even distribution of nuclear chromatin. In contrast, typical apoptotic cells had densely stained nuclei with a half-moon fluorescence shape located in the nucleus and cytoplasm, irregular nuclear shapes, and condensation and fragmentation of nuclear chromatin.

The results of cellular apoptotic rates are summarized in Table 2. Cells treated with *S. sonnei* alone were apoptotic in all sections sampled, with an apoptosis rate of 100% (Figure 3D). Cells treated with SLAP of M5-L and Q8-L alone were essentially normal, with apoptosis rates of only 5% (Figure 3B) and 10.67% (Figure 3C), respectively. These results indicate that SLAP exerted no effects on the normal growth processes of these cells. Cells infected with *S. sonnei* and co-incubated with SLAP of M5-L or Q8-L were only partially apoptotic with apoptosis rates of 37.67% (Figure 3E) and 43.67% (Figure 3F), respectively. These rates were lower than that of apoptosis rates of cells with *S. sonnei* alone, indicating that SLAP exerted a protective effect on cells invaded by *S. sonnei*.

Caspase-1 Activity Assays

Caspases are a family of cysteine-aspartic proteases that modulate apoptotic responses. In the absence of apoptotic stimuli, caspases exist in an inactive form as procaspases (Riedl and Salvesen, 2007). Therefore, evaluation of initiator caspase activation can be used to determine the apoptotic pathways involved. To assess the effects of SLAP of M5-L and Q8-L on caspase-1 activation after infection of intestinal epithelial cells with *S. sonnei*, we determined caspase-1 activity changes after infection. The SLAP of M5-L (Figure 4A) significantly inhibited caspase-1 activity as demonstrated

Figure 2. Inhibition rates resulting from varying concentrations of surface-layer associated proteins (SLAP) of *Lactobacillus paracasei* ssp. *paracasei* M5-L (A) and *Lactobacillus casei* Q8-L (B) on *Shigella sonnei*. Data are means $\pm$ SD of 3 independent experiments; *S. sonnei* alone served as a control [% *S. sonnei* prevented from adherence to HT-29 cells % (control – test)/control].

Figure 3. The surface-layer associated proteins (SLAP) of lactobacilli inhibited *Shigella sonnei*-induced human cell apoptosis. (A) Intact monolayer of uninfected HT-29 cells; (B) treatment of HT-29 cells with SLAP of *Lactobacillus paracasei* ssp. *paracasei* M5-L alone; (C) treatment of HT-29 cells with SLAP of *Lactobacillus casei* Q8-L alone; (D) treatment of HT-29 cells with *S. sonnei* alone; (E) HT-29 cells infected with *S. sonnei* and co-incubated with SLAP of M5-L; and (F) HT-29 cells infected with *S. sonnei* and co-incubated with SLAP of Q8-L. Arrows indicate cell apoptosis. Color version available online.

by the exclusion ($17.81 \pm 0.26\%$ reduction) and competition ($30.22 \pm 0.32\%$ reduction) assays compared with that observed in response to *S. sonnei* alone ($P < 0.05$). No decrease in caspase-1 activity by SLAP of M5-L was observed in the displacement assay compared with *S. sonnei* alone ($P > 0.05$). However, compared with *S. sonnei* alone, SLAP of Q8-L (Figure 4B) significantly inhibited caspase-1 activity in all 3 assays, with reductions of $42.19 \pm 0.28\%$, $30.87 \pm 0.73\%$, and $22.63 \pm 0.27\%$ observed in the exclusion, competition, and displacement assays, respectively ($P < 0.05$). These

findings demonstrate that SLAP of M5-L and Q8-L can reduce *S. sonnei*-induced apoptosis in HT-29 cells by inhibiting the activation of caspase-1 as revealed in all 3 assays for Q8-L and in the exclusion and competition assays for M5-L.

Distribution of ZO-1 and Occludin

Basal concentrations of ZO-1 and occludin antibodies were determined in a pretrial experiment by immunohistochemical analysis. The observation of brown portions distributed uniformly along the cell membrane indicated that optimal concentrations of ZO-1 and occludin antibodies were obtained with a 1:100 dilution. The results of this experiment revealed that the ZO-1 protein was visible in the cytoplasm and occludin was distributed along the outer membrane, which verified that the ZO-1 protein was located in the cell membrane and occludin located extracellularly (data not shown).

The SLAP of M5-L and Q8-L significantly increased the production of ZO-1 and occludin, whereas *S. sonnei* decreased production of ZO-1 compared with that of the control group. When cells infected with *S. sonnei* were co-incubated with the SLAP of M5-L and Q8-L, *S. sonnei*-induced ZO-1 and occludin distributions were inhibited compared with *S. sonnei*-infected cells alone (Figure 5).

Table 2. Apoptosis of HT-29 cells determined by Hoechst 33258 fluorescent analysis after co-incubation with *Shigella sonnei* and *Lactobacillus* SLAP (M5-L and Q8-L), which are illustrated in Figure 3

Treatment ¹	Apoptosis rate of HT-29 cells ² (%)
Control (uninfected HT-29 cells)	0
SLAP of M5-L	5 ± 2.65
SLAP of Q8-L	10.67 ± 2.52
<i>Shigella sonnei</i>	100
SLAP of M5-L + <i>S. sonnei</i>	37.67 ± 3.52
SLAP of Q8-L + <i>S. sonnei</i>	43.67 ± 3.51

¹SLAP = surface-layer associated protein; M5-L = *Lactobacillus paracasei* ssp. *paracasei* M5-L; Q8-L = *Lactobacillus casei* Q8-L.

²Data are means $\pm$ SD of 3 independent experiments.

DISCUSSION

The in vitro testing of lactobacilli-induced SLAP activity in simulated GIT conditions can provide a valuable means for predicting their actual in vivo activity under natural or clinical conditions following consumption. The SLAP from lactobacilli have been reported to exhibit good tolerance to digestive enzymes and show lower degrees of degradation in the intestinal tract than that of the stomach (Xiao, 2012), findings that agree with our current results. Sun et al. (2012) reported that SLAP could protect lactobacilli cells against the hos-

Figure 4. Function of surface-layer associated proteins (SLAP) of *Lactobacillus paracasei* ssp. *paracasei* M5-L (A) and *Lactobacillus casei* Q8-L (B) in caspase-1 activity assays. *S. sonnei* = *Shigella sonnei*. Data are means $\pm$ SD of 3 independent experiments. Letters (a–e) indicate differences between assays.

Figure 5. The surface-layer associated proteins (SLAP) of lactobacilli inhibited *Shigella sonnei*-induced ZO-1 and occludin redistribution. (A) Control group (uninfected HT-29 cells) for ZO-1; (B) treatment of HT-29 cells with SLAP of *Lactobacillus paracasei* ssp. *paracasei* M5-L alone; (C) treatment of HT-29 cells with SLAP of *Lactobacillus casei* Q8-L alone; (D) treatment of HT-29 cells with *S. sonnei* alone; (E) HT-29 cells infected with *S. sonnei* and co-incubated with SLAP of M5-L; (F) HT-29 cells infected with *S. sonnei* and co-incubated with SLAP of Q8-L; (G) control group (uninfected HT-29 cells) for occludin; (H) treatment of HT-29 cells with SLAP of M5-L alone; (I) treatment of HT-29 cells with SLAP of Q8-L alone; (J) treatment of HT-29 cells with *S. sonnei* alone; (K) HT-29 cells infected with *S. sonnei* and co-incubated with SLAP of M5-L; and (L) HT-29 cells infected with *S. sonnei* and co-incubated with SLAP of Q8-L. Magnification: 200 $\times$. Color version available online.

tile environment resulting from secretions of the small intestine. Our current results demonstrate that SLAP of M5-L were partially degraded under the simulated gastric conditions in our experiment. Accordingly, it would be necessary to use some type of microcapsule to protect SLAP of M5-L in the stomach to ensure their function within the intestinal tract. In contrast, the SLAP of Q8-L remained at 45 kDa under these simulated conditions, which suggests that they would have the capacity for transport into the intestine through the stomach. Although the use of lactobacilli-induced SLAP against pathogen infection has been widely studied, the functional mechanisms underlying these effects have received much less attention. It has been proposed that these surface proteins may alter the competitive binding of intestinal epithelial cells to prevent bacterial adhesion and invasion; therefore, we directed our

efforts at examining some of the potential mechanisms involved.

The findings of Xue et al. (2015) clearly showed that SLAP from *L. paracasei* M7 were responsible for their adherence to HT-29 cells. Moreover, they can inhibit the adhesion of *Salmonella* to HT-29 cells, as *Salmonella* was reduced by 42% in their exclusion assay experiment compared with that of controls. In our current experiments, we demonstrated that SLAP extracted from Q8-L and M5-L were ineffective in inhibiting the growth of *S. sonnei*, in agreement with results of Li et al. (2011). However, the SLAP of Q8-L and M5-L could directly inhibit *S. sonnei* adhesion to HT-29 cells as revealed in competition, exclusion, and displacement assays, although no obvious linear relationship was observed between inhibition rates and SLAP concentrations. Prophylactic use of SLAP may prove more effective than therapeutic use.

The SLAP of *L. acidophilus* ATCC 4356 can reduce apoptosis induced by *Salmonella typhimurium* SL1344, and this reduction in apoptosis is closely related to the time of application, with a maximal inhibitory effect observed at 36 h (Li et al., 2011). During pathogen-induced apoptosis, some critical factors are activated, such as caspase-1. Caspase-1 is a key executioner caspase in the proteolytic cascade that leads to apoptotic cell death, and it cleaves several structural proteins during the execution phase of apoptosis (Müller et al., 2009). In our experiment, we showed that *S. sonnei* was capable of invading epithelial cells, leading to cell apoptosis. Of greater significance were the findings that SLAP of Q8-L and M5-L in HT-29 cells inhibited this *S. sonnei*-induced apoptosis, a process that, in part, involves the capacity for SLAP to inhibit caspase-1 activation.

Resta-Lenert and Barrett (2003) reported that both *Streptococcus thermophilus* and *L. acidophilus* increased the resistance of HT-29 and Caco-2 cells to membrane barrier disruption caused by the pathogen, while maintaining or increasing the phosphorylation of TJ proteins such as actinin and occludin. In those studies, the culture media and heat-inactivated bacteria did not change cell membrane resistance. Similarly, *Lactobacillus rhamnosus* and *Bifidobacterium* increased phosphorylation of ZO-1 and occludin and increased epithelial resistance (Mathias et al., 2010). These increases in resistance are accompanied by a decrease in claudin-2 protein expression and an increase in ZO-1 and occludin protein production. Such results suggest some of the basis for their effects in reducing intestinal cell permeability and improving disease-induced colitis. Our current results are closely related to these findings. Specifically, we show that the SLAP of M5-L and Q8-L not only provide partial protection of intestinal

epithelial cells but also promote the production of ZO-1 and occludin in these cells.

CONCLUSIONS

Our results provide new insights into the function of SLAP of Q8-L and M5-L, which can inhibit the adherence of *S. sonnei*, as revealed by exclusion, competition, and displacement assays. We demonstrated that SLAP of Q8-L and M5-L may be directly involved in this process by reducing downstream caspase-1 activation, thus modulating apoptotic cell damage. In addition, these SLAP can partly protect intestinal epithelial cells and promote the production of ZO-1 and occludin in cells. Uncovering the mechanism involved could enable novel approaches for the use of SLAP in antagonizing *S. sonnei* infection.

ACKNOWLEDGMENTS

This work was financially supported by National Natural Science Foundation of China (31301515), Natural Science Foundation of Heilongjiang Province (C201433), China Postdoctoral Science Foundation (2012M510093, 2013T60382), and the Fundamental Research Funds for the Central Universities (Grant no. HIT.NSRIF.2012082; Harbin Institute of Technology).

REFERENCES

- Allen, S., J. Sotos, M. J. Sylte, and C. J. Czuprynski. 2001. Use of Hoechst 33342 staining to detect apoptotic changes in bovine mononuclear phagocytes infected with *Mycobacterium avium* ssp. *paratuberculosis*. Clin. Diagn. Lab. Immunol. 8:460–464.
- Anderson, M., P. J. Sansonetti, and B. S. Marteyn. 2016. *Shigella* diversity and changing landscape: Insights for the twenty-first century. Front. Cell. Infect. Microbiol. 6:45.
- Avall-Jääskeläinen, S., A. Lindholm, and A. Palva. 2003. Surface display of the receptor-binding region of the *Lactobacillus brevis* S-layer protein in *Lactococcus lactis* provides nonadhesive lactococci with the ability to adhere to intestinal epithelial cells. Appl. Environ. Microbiol. 69:2230–2236.
- Beveridge, T. J., P. H. Pouwels, M. Sára, A. Kotiranta, K. Lounatmaa, K. Kari, E. Kerosuo, M. Haapasalo, E. M. Egelseer, I. Schocher, U. B. Sleytr, L. Morelli, M. L. Callegari, J. F. Nomellini, W. H. Bingle, J. Smit, E. Leibovitz, M. Lemaire, I. Miras, S. Salamitous, P. Béguin, H. Ohayon, P. Gounon, M. Matuschek, and S. F. Koval. 1997. Functions of S-layers. FEMS Microbiol. Rev. 20:99–149.
- Breuer, U. 2009. *Lactobacillus* molecular biology. From genomics to probiotics. Biotechnol. J. 4:769.
- Bron, P. A., P. van Baarlen, and M. Kleerebezem. 2011. Emerging molecular insights into the interaction between probiotics and the host intestinal mucosa. Nat. Rev. Microbiol. 10:66–78.
- Candela, M., F. Perna, P. Carnevali, B. Vitali, R. Ciati, and P. Gionchetti. 2008. Interaction of probiotic *Lactobacillus* and *Bifidobacterium* strains with human intestinal epithelial cells: Adhesion properties, competition against enteropathogens and modulation of IL-8 production. Int. J. Food Microbiol. 125:286–292.
- Charteris, W. P., P. M. Kelly, L. Morelli, and J. K. Collins. 1998. Development and application of an *in vitro* methodology to determine the transit tolerance of potentially probiotic *Lactobacillus* and *Bi-*

- fidobacterium* species in the upper human gastrointestinal tract. J. Appl. Microbiol. 84:759–768.
- Chen, X. Y., J. J. Xu, J. B. Shuai, J. S. Chen, Z. F. Zhang, and W. H. Fang. 2007. The S-layer proteins of *Lactobacillus crispatus* strain ZJ001 is responsible for competitive exclusion against *Escherichia coli* O157:H7 and *Salmonella typhimurium*. Int. J. Food Microbiol. 115:307–312.
- Golowcyc, M. A., P. Mobili, G. L. Garrote, A. G. Abraham, and G. L. De Antoni. 2007. Protective action of *Lactobacillus kefir* carrying S-layer protein against *Salmonella enterica* serovar Enteritidis. Int. J. Food Microbiol. 118:264–273.
- Granato, D., F. Perotti, I. Masserey, M. Rouvet, M. Golliard, A. Servin, and D. Brassart. 1999. Cell surface-associated lipoteichoic acid acts as an adhesion factor for attachment of *Lactobacillus johnsonii* La1 to human enterocyte-like Caco-2 cells. Appl. Environ. Microbiol. 65:1071–1077.
- Jakava-Viljanen, M., and A. Palva. 2007. Isolation of surface (S) layer protein carrying *Lactobacillus* species from porcine intestine and faeces and characterization of their adhesion properties to different host tissues. Vet. Microbiol. 124:264–273.
- Khang, Y. H., H. Y. Park, Y. S. Jeong, J. A. Kim, and Y. H. Kim. 2009. Recombinant S-layer proteins of *Lactobacillus brevis* mediating antibody adhesion to calf intestine alleviated neonatal diarrhea syndrome. J. Microbiol. Biotechnol. 19:511–519.
- Li, P. C., X. L. Ye, and Q. Yang. 2012. Antagonistic activity of *Lactobacillus acidophilus* ATCC 4356 S-layer protein on *Salmonella enterica* ssp. *enterica* serovar *Typhimurium* in Caco-2 cells. Ann. Microbiol. 62:905–909.
- Li, P. C., Y. Y. Yin, Q. H. Yu, and Q. Yang. 2011. *Lactobacillus acidophilus* S-layer protein-mediated inhibition of *Salmonella*-induced apoptosis in Caco-2 cells. Biochem. Biophys. Res. Commun. 409:142–147.
- Lortal, S., J. V. Heijenoort, K. Gruber. And, and U. B. Sleytr. 1992. S-layer of *Lactobacillus helveticus* ATCC 12046: Isolation, chemical characterization and re-formation after extraction with lithium chloride. J. Gen. Microbiol. 138:611–618.
- Lu, R. Y., W. X. Yang, and Y. J. Hu. 2014. The role of epithelial tight junctions involved in pathogen infections. Mol. Biol. Rep. 41:6591–6610.
- Maragkoudakis, P. A., C. Miaris, P. Rojez, N. Manalis, F. Magkanari, G. Kalantzopoulos, and E. Tsakalidou. 2006. Production of traditional Greek yoghurt using *Lactobacillus* strains with probiotic potential as starter adjuncts. Int. Dairy J. 16:52–60.
- Mathias, A., M. Duc, L. Favre, J. Benyacoub, S. Blum, and B. Cortésy. 2010. Potentiation of polarized intestinal Caco-2 cell responsiveness to probiotics complexed with secretory IgA. J. Biol. Chem. 285:33906–33913.
- Müller, A. J., C. Hoffmann, M. Galle, A. V. D. Broeke, M. Heikenwalder, L. Falter, B. Misselwitz, M. Kremer, R. Beyaert, and W. D. Hardt. 2009. The *S.typhimurium* effector SopE induces caspase-1 activation in stromal cells to initiate gut inflammation. Cell Host Microbe 6:125–136.
- Ramiah, K., C. A. van Reenen, and L. M. T. Dicks. 2008. Surface-bound proteins of *Lactobacillus plantarum* 423 that contribute to adhesion of Caco-2 cells and their role in competitive exclusion and displacement of *Clostridium sporogenes* and *Enterococcus faecalis*. Res. Microbiol. 159:470–475.
- Resta-Lenert, S., and K. E. Barrett. 2003. Live probiotics protect intestinal epithelial cells from the effects of infection with enteroinvasive *Escherichia coli* (EIEC). Gut 52:988–997.
- Riedl, S. J., and G. S. Salvesen. 2007. The apoptosome: Signalling platform of cell death. Nat. Rev. Mol. Cell Biol. 8:405–413.
- Servin, A. L. 2004. Antagonistic activities of lactobacilli and bifidobacteria against microbial pathogens. FEMS Microbiol. Rev. 28:405–440.
- Singh, R., S. Pervin, G. Y. Wu, and G. Chaudhuri. 2001. Activation of caspase-3 activity and apoptosis in MDA-MB-468 cells by N ω -hydroxy-L-arginine, an inhibitor of arginase, is not solely dependent on reduction in intracellular polyamines. Carcinogenesis 22:1863–1869.
- Sleytr, U. B. 1976. Self-assembly of the hexagonally and tetragonally arranged subunits of bacterial surface layers and their reattachment to cell walls. J. Ultrastruct. Res. 55:360–377.
- Sleytr, U. B., B. Schuster, E. M. Egelseer, and D. Pum. 2014. S-layers: Principles and applications. FEMS Microbiol. Rev. 38:823–864.
- Smit, E., F. Oling, R. Demel, B. Martinez, and P. H. Pouwels. 2001. The S-layer protein of *Lactobacillus acidophilus* ATCC 4356: Identification and characterisation of domains responsible for S-protein assembly and cell wall binding. J. Mol. Biol. 305:245–257.
- Sun, Z. L., L. H. Huang, J. Kong, S. M. Hu, X. W. Zhang, and W. B. Kong. 2012. *In vitro* evaluation of *Lactobacillus crispatus* K313 and K243: high-adhesion activity and anti-inflammatory effect on *Salmonella braenderup* infected intestinal epithelial cell. Vet. Microbiol. 159:212–220.
- Sun, Z. L., J. Kong, S. M. Hu, and W. T. Kong. 2013. Characterization of a S-layer protein from *Lactobacillus crispatus* K313 and the domains responsible for binding to cell wall and adherence to collagen. Appl. Microbiol. Biotechnol. 97:1941–1952.
- Šušković, J., B. Kos, J. Beganovic, A. L. Pavunc, K. Habjanič, and S. Matošić. 2010. Antimicrobial activity-the most important property of probiotic and starter lactic acid bacteria. Food Technol. Biotechnol. 48:296–307.
- Tran Van Nhieu, G., R. Bourdet-Sicard, G. Duménil, A. Blocker, and P. J. Sansonetti. 2000. Bacterial signals and cell responses during *Shigella* entry into epithelial cells. Cell. Microbiol. 2:187–193.
- Xiao, R. 2012. Screening of the *Lactobacillus brevis* M8 and cloning and expression analysis of its surface layer gene slpM. PhD Diss. Hunan Agricultural University, Hunan, China.
- Xue, C. H., L. W. Zhang, R. B. Fan, S. M. Wang, H. B. Li, X. Luo, W. L. Liu, and W. Song. 2015. *Salmonella* infection and mediated inhibition of *Salmonella*-induced apoptosis. Eur. Food Res. Technol. 240:1–7.
- Zhang, W. M., H. F. Wang, J. X. Liu, Y. H. Zhao, K. Gao, and J. Zhang. 2013. Adhesive ability means inhibition activities for *lactobacillus* against pathogens and S-layer protein plays an important role in adhesion. Anaerobe 22:97–103.
- Zhang, Y. C., X. L. Xiang, Q. H. Lu, L. W. Zhang, F. Ma, and L. Wang. 2016. Adhesions of extracellular surface-layer associated proteins in *Lactobacillus* M5-L and Q8-L. J. Dairy Sci. 99:1011–1018.
- Zhang, Y. C., L. W. Zhang, D. Ming, H. X. Yi, C. Guo, and Y. F. Tuo. 2011. Antimicrobial activity against *Shigella sonnei* and probiotic properties of wild *lactobacilli* from fermented food. Microbiol. Res. 167:27–31.
- Zhang, Y. C., L. W. Zhang, Y. F. Tuo, C. F. Guo, H. X. Yi, J. Y. Li, X. Han, and M. Du. 2010. Inhibition of *Shigella sonnei* adherence to HT-29 cells by lactobacilli from Chinese fermented food and preliminary characterization of S-layer protein involvement. Res. Microbiol. 161:667–672.