Adhesions of extracellular surface-layer associated proteins in *Lactobacillus* M5-L and Q8-L

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ABSTRACT

Surface-layer associated proteins (SLAP) that envelop *Lactobacillus paracasei* ssp. *paracasei* M5-L and *Lactobacillus casei* Q8-L cell surfaces are involved in the adherence of these strain to the human intestinal cell line HT-29. To further elucidate some of the properties of these proteins, we assessed the yields and expressions of SLAP under different incubation conditions. An efficient and selective extraction of SLAP was obtained when cells of *Lactobacillus* were treated with 5 M LiCl at 37°C in aerobic conditions. The SLAP of *Lactobacillus* M5-L and Q8-L in cell extracts were visualized by SDS-PAGE and identified by Western blotting with sulfo-*N*-hydroxysuccinimide-biotin-labeled HT-29 cells as adhesion proteins. Atomic force microscopy contact imaging revealed that *Lactobacillus* strains M5-L and Q8-L normally display a smooth, homogeneous surface, whereas the surfaces of M5-L and Q8-L treated with 5 M LiCl were rough and more heterogeneous. Analysis of adhesion forces revealed that the initial adhesion forces of 1.41 and 1.28 nN obtained for normal *Lactobacillus* M5-L and Q8-L strains, respectively, decreased to 0.70 and 0.48 nN, respectively, following 5 M LiCl treatment. Finally, the dominant 45-kDa protein bands of *Lactobacillus* Q8-L and *Lactobacillus* M5-L were identified as elongation factor Tu and surface antigen, respectively, by liquid chromatography-tandem mass spectrometry.

Key words: *Lactobacillus*, surface-layer associated proteins, adhesion, identification

INTRODUCTION

The genus *Lactobacillus* is a common inhabitant of the intestinal tracts of humans and animals (Walter et al., 2003). The ability for *Lactobacillus* to adhere to epithelial tissues is key to its success as an agent capable

of maintaining a persistent colonization within mammalian intestines and other tissues (Hynönen et al., 2002). Recently, several *Lactobacillus* species have been shown to exhibit strong adherence to intestinal epithelium cells, and surface (S)-layer proteins were identified as being involved in this adhesion process (Chen et al., 2007a; Khang et al., 2009; Sun et al., 2013) and in the inhibition of *Salmonella*-induced reorganization of the cytoskeleton (Li et al., 2011). The peptidoglycan layer of the *Lactobacillus* cell wall is covered with a variety of substances, the most notable of these being (lipo)teichoic acids, neutral and acidic polysaccharides, and (surface) proteins (Delcour et al., 1999). Bacterial S-layers are crystalline arrays of self-assembling, proteinaceous subunits called S-layer proteins (Slp), with molecular masses ranging from 40 to 200 kDa. In addition to Slp, anchorless multifunctional proteins have also been associated with lactobacilli (Bergonzelli et al., 2006; Granato et al., 2004). The elongation factor Tu (EF-Tu) is an intracellular protein that functions in protein synthesis and folding. Surface enzymes such as the cell surface proteinase PrtB of *Lactobacillus delbrueckii* ssp. *bulgaricus* have been described that mediate *Lactobacillus* adhesion to mucin and human epithelial cells (Gilbert et al., 1996).

Lactobacillus paracasei ssp. *paracasei* M5-L and *Lactobacillus casei* Q8-L, strains that can survive passage through the gastrointestinal tract and exert antimicrobial activity against 3 pathogens, were found to adhere effectively to HT-29 cells (Zhang et al., 2011). In addition, the M5-L and Q8-L strains possess the ability to inhibit *Shigella sonnei* adherence to HT-29 cells, and surface-layer associated proteins (SLAP) are involved in this adhesion inhibition (Zhang et al., 2010).

The main objective of this study was to evaluate some of these properties of SLAP. Our specific aims were to (1) conduct an assay of the effects of incubation conditions on the yield and expressions of SLAP; (2) evaluate SLAP adhesion to HT-29 cells; (3) determine SLAP functions on adhesion forces of M5-L and Q8-L; and (4) identify SLAP using liquid chromatography-tandem MS (LC-MS/MS). The information thus

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obtained will provide important new information regarding the role of SLAP in probiotic activities.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions

Lactobacillus paracasei ssp. *paracasei* M5-L (hereafter M5-L) was isolated from koumiss made in local households in Sinkiang, and *L. casei* Q8-L (hereafter Q8-L) was isolated from yak yogurt made in local households in Qinghai (Zhang et al., 2010). Both strains were stored in our laboratory and cultured in de Man, Rogosa, and Sharpe (MRS) broth (Difco/Becton Dickinson Co., Franklin Lakes, NJ) under aerobic conditions at 37°C.

Extraction of SLAP

The SLAP of M5-L and Q8-L were extracted with 5 M LiCl as described previously (Lortal et al., 1992; Smit et al., 2001). Briefly, after a 16-h incubation period, cells from 500 mL of MRS broth 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, Beijing, China) at 150 rpm and 37°C for 60 min to extract proteins bound noncovalently. The supernatant was harvested by centrifugation, dialyzed extensively against distilled water that was changed every 2 h for the first 8 h to remove any LiCl from the liquid, concentrated with PEG-8000 (Amresco, Solon, OH), and stored at -20°C until lyophilized.

SDS-PAGE Analysis

Ten milliliters of supernatant containing SLAP was precipitated with 2 volumes of cold acetone (2 h at -20°C) and centrifuged ($10,000 \times g$ at 4°C for 10 min). The precipitate was resuspended in 100 μ L of loading buffer, boiled for 5 min, and analyzed by SDS-PAGE as described by Laemmli (1970).

Effects of Incubation Conditions on Yield and Expression of SLAP

Strains M5-L and Q8-L were grown in MRS broth and incubated at different temperatures (25°C, 37°C, or 42°C) under aerobic and anaerobic atmospheric conditions. Extraction of SLAP from M5-L and Q8-L was as described above. Concentrations of S-layer proteins were estimated using the Coomassie Blue Staining Kit (Beyotime Institute of Biotechnology, Jiangsu, China).

Expression of SLAP was analyzed by SDS-PAGE as described by Laemmli (1970).

HT-29 Cell Cultures

The HT-29 cells were obtained from the Cancer Institute of the Chinese Academy of Medical Science (Beijing). The cells were cultured in complete RPMI-1640 medium (Hyclone, Logan, UT) supplemented with 10% (vol/vol) inactivated (56°C, 30 min) fetal bovine serum (Sijiqing Co. Ltd., Zhejiang, China), in an incubator (HEPA class 100, Thermo Scientific HyClone, Waltham, MA) with 5% CO₂ and 95% air at 37°C. The culture medium was changed daily until HT-29 monolayers were at 80 to 90% confluence, and HT-29 monolayers were then washed twice with PBS (pH 7.4) before use in the experiments.

Sulfo-NHS-Biotin-Labeled HT-29 Cell Preparation

The HT-29 cells were conjugated with sulfo-*N*-hydroxysuccinimide-biotin (B-NHS, Sigma, St. Louis, MO) using methods described previously (Rojas et al., 2002; Chen et al., 2007b) with the following modifications. The concentration of HT-29 cells was adjusted to 2×10^7 cells/mL. Two milliliters of B-NHS (10 mM) was added to 10 mL of HT-29 cells, so that the final concentration of the B-NHS in the HT-29 cells was 2 mM. This mixture was stirred gently at room temperature for 30 min. After extensive dialysis in PBS buffer (pH 8.0) with 100 mM glycine and 3 washes to inhibit unlabeled-B-NHS, the HT-29 cells were stored at 4°C until use in the Western blot assay.

Western Blot Assay of SLAP Adhesion

The SLAP of strains M5-L and Q8-L were prepared as described above. After SDS-PAGE, the separated surface proteins of M5-L and Q8-L with molecular weights of 45 kDa were electrophoretically transferred to an Immobilon polyvinylidene difluoride (PVDF; Millipore, Billerica, MA) membrane at 20 V for 50 min. The membrane was then washed 3 times (5 min per wash) with PBS and incubated with B-NHS-labeled HT-29 cells at 37°C in 5% CO₂ for 1 h. After washing 3 times with PBS (pH 7.4; 10 min per wash), 1:500 horseradish peroxidase-avidin (Beijing Bo Ao Sen Biotechnology Co. Ltd., Beijing, China) was added and incubated for 1 h. The membrane was agitated at room temperature for 30 min, and then washed 3 times with PBS (pH 7.4). The substrate 3,3'-diaminobenzidine (Beijing Solarbio Biotechnology Co. Ltd., Beijing, China) was added. The reaction was stopped by wash-

ing with distilled water when the specific protein band appeared (Sengupta et al., 1992).

Atomic Force Microscopy Analysis

The M5-L and Q8-L strains were treated with 5 M LiCl as described above, and then tested for force of adhesion and atomic force microscopy (AFM) contact imaging. The cells were washed 3 times with PBS and adjusted to 2×10^7 cfu/mL. The cells of M5-L and Q8-L were washed 3 times with PBS (pH 7.0) as a control. Before AFM (MultiMode IIIa, Bruker Corp., Billerica, MA) analysis, the LiCl-treated M5-L and Q8-L were adhered to a poly-L-lysine-covered glass slide. Bacterial adhesion was performed at room temperature ($22 \pm 1^\circ\text{C}$) by depositing a drop of the bacterial suspension buffered at pH 7 on a poly-L-lysine-coated glass slide (Booster, Wuhan, China) and then incubated for up to 1 h at room temperature. The slides were washed with ultrapure water (MilliQ, Millipore) immediately before use. The AFM measurements were performed at 20°C in 10 mM KH_2PO_4 buffer adjusted to pH 7. Contact mode images were taken in a constant force mode with the applied force maintained at <1 nN. The scan rate varied between 1.0 and 2.5 Hz, and Si_3N_4 microfabricated Nanoprobe cantilevers (Digital Instruments, MultiMode IIIa, Bruker Corp., Billerica, MA) with a nominal spring constant of 0.06 N/m were used. The AFM tips were plasma-treated immediately before use (Schär-Zammaretti and Ubbink, 2003).

Identification of Adhesion SLAP from M5-L and Q8-L

Bands representing SLAP with molecular weights of 45 kDa were excised from gels, purified, and prepared for MS by the in-gel tryptic digest procedure described by Guillot et al. (2003). Intact protein bands were digested with sequencing-grade modified trypsin (Promega, Madison, WI). A Waters API Q-TOF Ultima

mass spectrometer fitted with a Waters CapLC system was used (Waters, Milford, MA). Tryptic peptides were separated on an Atlantis dC18 column (Waters). Samples of 5 mL were injected at a flow rate of 200 nL/min. All experiments were performed in triplicate. Protein identification was achieved by searching the MS/MS spectra against the MASCOT database using the Mascot search engine, MS/MS ion search option (<http://www.matrixscience.com>) against the nonredundant protein National Center for Biotechnology Information (NCBI) database (<http://www.ncbi.nlm.nih.gov/>). Independent experiments were replicated 3 times.

RESULTS

Effects of Incubation Conditions on SLAP Production

The yield and SDS-PAGE of SLAP from M5-L and Q8-L at different temperatures are summarized in Table 1 and Figure 1. Greater concentrations of SLAP were extracted from M5-L grown under aerobic culture conditions at 42°C (0.81 mg/mL) compared with 25°C (0.46 mg/mL) or 37°C (0.42 mg/mL). A concentration of 0.57 mg/mL was obtained in M5-L under anaerobic conditions at 37°C . The results of SDS-PAGE showed that the predominant bands were 45 kDa and distinct, with fewer other proteins bands being present in extractions performed at 37°C (Figure 1, lane 2) compared with other incubation conditions. The SLAP yields of Q8-L in anaerobic conditions were less dependent upon temperature, with yields of 0.70 mg/mL at 25°C , 0.76 mg/mL at 37°C (aerobic), 0.76 mg/mL at 42°C , and 0.80 mg/mL at 37°C (anaerobic; Table 1). No significant differences were present among these yields of SLAP at different temperature for Q8-L. However, results of SDS-PAGE revealed that the predominant bands were more distinct when the M5-L and Q8-L strains were cultured at 37°C .

Table 1. Total surface-layer protein content extracted under different culture conditions

<i>Lactobacillus</i> ¹	Culture conditions	Optical density at 595 nm	Total protein content (mg/mL)
M5-L	Aerobic at 25°C	0.83 ± 0.03	0.46 ± 0.01^c
M5-L	Aerobic at 37°C	0.75 ± 0.00	0.42 ± 0.01^c
M5-L	Aerobic at 42°C	1.44 ± 0.03	0.81 ± 0.00^a
M5-L	Anaerobic 37°C	1.02 ± 0.00	0.57 ± 0.01^b
Q8-L	Aerobic at 25°C	1.25 ± 0.00	0.70 ± 0.01^{ab}
Q8-L	Aerobic at 37°C	1.34 ± 0.01	0.76 ± 0.01^{ab}
Q8-L	Aerobic at 42°C	1.35 ± 0.04	0.76 ± 0.01^{ab}
Q8-L	Anaerobic 37°C	1.42 ± 0.01	0.80 ± 0.01^a

^{a-c}Values within a column with different letters indicate a significant difference ($P < 0.05$).

¹Strains: M5-L = *Lactobacillus paracasei* ssp. *paracasei* M5-L; Q8-L = *Lactobacillus casei* Q8-L.

Adhesion of SLAP to HT-29 Cells

After SDS-PAGE (Figure 2B: M5-L in lane 1 and Q8-L in lane 2) and subsequent Western blotting using B-NHS-labeled HT-29 cells (Figure 2A), the extraction SLAP of *Lactobacillus* M5-L and Q8-L cells yielded a molecular mass of 45 kDa under denaturing conditions (M5-L in 1 and Q8-L in 2). The 45-kDa SLAP were dominant in cell extracts compared with proteins of other molecular masses and could be specifically detected by their binding to HT-29 cells. A stronger-reacting band representing SLAP and HT-29 cells was observed for strain Q8-L than for strain M5-L.

AFM Contact Imaging

Contact-mode images were taken with minimal force in retraction mode. From the error-signal deflection mode images (Figure 3), it was possible to obtain information on the dimensions of the microorganisms and qualitative data on the structure of their surfaces. Whereas M5-L (Figure 3A) and Q8-L (Figure 3C) normally display a smooth, homogeneous surface, after treatment of the cultures with 5 M LiCl, a rough and more heterogeneous surface of M5-L (Figure 3B) and Q8-L (Figure 3D) was observed.

AFM Analysis of Adhesion Force

An additional parameter assessed was a comparison of the force-distance curves of M5-L and Q8-L with (Figure 4 A and C) and without (Figure 4 B and D) SLAP. An initial observation of adhesion forces of M5-L and Q8-L with SLAP revealed values of 1.41 and 1.28 nN,

Figure 1. Sodium dodecyl sulfate-PAGE of surface-layer protein extracted under different culture conditions. M = molecular mass marker; lane 1 = *Lactobacillus paracasei* ssp. *paracasei* M5-L (M5-L), 25°C, aerobic; lane 2 = M5-L, 37°C, aerobic; lane 3 = M5-L, 42°C, aerobic; lane 4 = M5-L, 37°C, anaerobic; lane 5 = *Lactobacillus casei* Q8-L (Q8-L), 25°C, aerobic; lane 6 = Q8-L, 37°C, aerobic; lane 7 = Q8-L, 42°C, aerobic; lane 8 = Q8-L, 37°C, anaerobic. Arrows indicate target 45-kDa bands.

Figure 2. Western blot analysis of surface-layer associated proteins (SLAP) of *Lactobacillus paracasei* ssp. *paracasei* M5-L and *Lactobacillus casei* Q8-L. The SLAP of M5-L and Q8-L were Western blotted using sulfo-*N*-hydroxysuccinimide-biotin-labeled HT-29 cells (panel A: 1 = M5-L; 2 = Q8-L) after SDS-PAGE (panel B: lane 1 = M5-L and lane 2 = Q8-L). Arrows indicate target 45-kDa bands.

respectively. Treatment with 5 M LiCl significantly affected these adhesion forces as M5-L and Q8-L without SLAP showed values of 0.70 and 0.48 nN, respectively. Additionally, the force-distance curve of M5-L and Q8-L shows a sharp minimum, which strongly suggests that separation of the bacterial surface from AFM tip proceeds via a single unbinding event.

Identification of the Adhesion-SLAP from M5-L and Q8-L

Protein identification was performed using peptide mass fingerprinting and automated MS/MS analysis of major ions in the matrix with assisted laser desorption/ionization. The dominant protein bands recorded for M5-L (Figure 2B: lane 1) and Q8-L (Figure 2B: lane 2) were excised from the SDS gel, purified and identified by MS. The dominant 45-kDa protein band for M5-L was identified as a surface antigen (SA) described for *L. casei* (score of 4,292) with a theoretical molecular mass of 41.95 kDa (Table 2). The Q8-L cell surface-extracted protein band with a theoretical molecular mass of 43.55 kDa was identified as EF-Tu, described for *Lactobacillus plantarum* (score of 443; Table 3). Molecular sizes of all SLAP corresponded to respective matches present in the MASCOT database, confirming the identity of the proteins.

DISCUSSION

Temperature and culture conditions are major factors influencing SLAP production in *Lactobacillus*. Efficient and selective extraction of SLAP was obtained when cultures were treated with 5 M LiCl at 37°C under aerobic conditions. Such conditions might perturb electrostatic interactions between certain surface-associated proteins and the cell envelope of the bacterium, allowing for

extraction of proteins with enhanced purity from the cell surface. In addition, attachment of some surface proteins was previously shown to be pH-dependent, enabling release into the bacterial surroundings at a neutral or slightly alkaline pH (Antikainen et al., 2007).

Western blotting has been used previously to characterize adhesion-associated proteins in protein mixtures (Lortal et al., 1992; Rojas et al., 2002; Chen et al., 2007b). With this technique, 29- and 61.8-kDa adhesion-promoting proteins, present on the cell surface of *L. fermentum* 104R and *Lactobacillus* L15, respectively, were isolated and characterized from cell extracts. Using the same method, we successfully identified and characterized an adhesion-associated protein with a molecular mass of 45 kDa in *Lactobacillus* strains M5-L and Q8-L as extracted from the cell surface. Recently, S-layer proteins of some *Lactobacillus* strains have been shown to function as key adhesion factors of the cell

and could play a role in probiotic activity (Wang et al., 2008).

The molecular sizes of the M5-L and Q8-L proteins corresponded with matches present in the MASCOT database and were identified as EF-Tu and SA; both may be regarded as multifunctional proteins that assist *Lactobacillus* M5-L and Q8-L in adapting to changes in their environment. Removal of surface-bound proteins reduced adherence of M5-L and Q8-L strains to HT-29 cells by 50%, suggesting that EF-Tu and SA may function as adhesion agents in M5-L and Q8-L (Zhang et al., 2011). The EF-Tu protein binds to guanosine triphosphate and is active during protein synthesis (Gaucher, et al., 2001). In lactobacilli, EF-Tu may function as an envelope-associated protein, which may be released from cells during osmotic shock (Nakamura et al., 1997). In the case of *Lactobacillus johnsonii* La1 (NCC 533), EF-Tu mediates attachment of the strain to mu-

Figure 3. Atomic force microscopy deflection images showing the surface morphology of the *Lactobacillus* strains. The microorganisms were adhered to a poly-L-lysine-covered substrate. Imaging was performed in a 10 mM KH_2PO_4 buffer at pH 7. (A) *Lactobacillus paracasei* ssp. *paracasei* M5-L (M5-L); (B) M5-L without surface (S)-layer proteins; (C) *Lactobacillus casei* Q8-L (Q8-L); (D) Q8-L without S-layer proteins. *Lactobacillus* M5-L (A) and Q8-L (C) normally display a smooth, homogeneous surface, whereas *Lactobacillus* M5-L (B) and Q8-L (D) showed a heterogeneous and rough surface when treated with 5 M LiCl.

Table 2. Proteins identified on the surface of *Lactobacillus paracasei* ssp. *paracasei* M5-L

Protein	Microorganism	Accession no. ¹	Score ²	Mass ³	AA matches	Sequences ⁴	emPAI ⁵
Surface antigen	<i>Lactobacillus casei</i>	gi 489671527	4,292	41.95	295 (209)	15 (12)	2.34
<i>N</i> -Acetylmuramoyl-L-alanine amidase	<i>L. casei</i> group	gi 489675024	838	46.94	65 (44)	19 (16)	2.41
Phosphopyruvate hydratase	<i>L. casei</i> ATCC 334	gi 116494476	384	47.06	46 (24)	17 (13)	1.92
Lysozyme	<i>L. casei</i> group	gi 489683400	291	72.02	16 (11)	9 (7)	0.42
L,D-transpeptidase	<i>L. casei</i>	gi 489680926	243	98.11	29 (16)	10 (6)	0.25

¹Protein sequence GenInfo Identifier (GI) number (<http://www.ncbi.nlm.nih.gov/protein>).
²MOWSE (Molecular Weight Search) score resulting from the ion MS/MS search against the nonredundant NCBI protein database (<http://www.ncbi.nlm.nih.gov/protein>). All scores were statistically significant ($P < 0.05$).
³Theoretical molecular mass.
⁴Tandem MS peptides whose fragmentation allowed the identification of the protein. AA matches>55 (values in parentheses) were statistically significant ($P < 0.05$).
⁵Protein abundant identification by expectation-maximization algorithm.

cus and Caco-2 cells (Granato et al., 2004). The EF-Tu protein of *L. johnsonii* NCC 533 shares high homology (84%) with the EF-Tu protein isolated from other *L. plantarum* strains, suggesting that they may have the

same function. Surface enzymes such as GAPDH and triosephosphate isomerase (TPI) are multifunctional proteins that are released from the cell to assist the organism in adapting to environmental changes and

Figure 4. Force–distance (*Z*) curves showing the interactions of the atomic force microscopy tip with *Lactobacillus paracasei* ssp. *paracasei* M5-L (M5-L); (B) M5-L without surface (S)-layer proteins; (C) *Lactobacillus casei* Q8-L (Q8-L); (D) Q8-L without S-layer proteins.

Table 3. Proteins identified on the surface of *Lactobacillus casei* Q8-L

Protein	Microorganism	Accession no. ¹	Score ²	Mass ³	AA matches	Sequences ⁴	emPAI ⁵
Elongation factor Tu	<i>L. casei</i> BL23	B3WE38	2,143	43.55	113 (82)	21 (19)	17.67
Enolase	<i>L. casei</i> ATCC 334	Q03AK4	1,772	47.06	71 (58)	25 (20)	9.01
Phosphoglycerate kinase	<i>L. casei</i> BL23	B3WCW5	1,149	42.21	63 (46)	23 (15)	4.67
30S ribosomal protein S2	<i>L. casei</i> BL23	B3WES8	1,013	29.53	57 (35)	16 (12)	7.46
L-Lactate dehydrogenase	<i>L. casei</i>	P00343	1,001	35.51	44 (33)	13 (11)	6.12

¹Protein sequence GenInfo Identifier (GI) number (<http://www.ncbi.nlm.nih.gov/protein>).

²MOWSE (Molecular Weight Search) score resulting from the ion MS/MS search against the nonredundant NCBI protein database (<http://www.ncbi.nlm.nih.gov/protein>). All scores were statistically significant ($P < 0.05$).

³Theoretical molecular mass.

⁴Tandem MS peptides whose fragmentation allowed the identification of the protein. AA matches > 55 (values in parentheses) were statistically significant ($P < 0.05$).

⁵Protein abundant identification by expectation-maximization algorithm.

can function as moonlighting proteins, which mediating microbe–host interactions in lactic acid bacteria (Kinoshita et al., 2008; Ramiah et al., 2008; Sánchez et al., 2009; Katakura et al., 2010). Interestingly, we initially found SA, an adhesion protein, in *Lactobacillus* M5-L.

Atomic force microscopy was used to evaluate the surface structure, interactions, and resiliency of the bacterial cell wall beginning at nanometer-length scales and continuing upward. Models of the outer layers of the bacterial cell wall have been presented previously (Schär-Zammaretti and Ubbink, 2003). The surface of M5-L lacked any fuzzy or heterogeneous nature at the scales probed in the AFM experiments, which suggests the absence of extended, loosely cross-linked polymer networks on the outside of the cell wall. When treated with 5 M LiCl, M5-L and Q8-L surfaces acquired a rough and patchy nature due to the extended surface protein layer being laterally repositioned. By combining these deflection image analyses, we inferred that the surface consists of a heterogeneous polymeric network, most likely composed of a compact protein layer.

Confirmation of these surface structures emerging from the deflection images was provided by the force–distance curves obtained on the same bacterial surfaces. Again, we found major qualitative differences between M5-L and Q8-L when treated with 5 M LiCl. In contrast to M5-L, Q8-L showed clear adhesion peaks upon retraction of the AFM tip from the bacterial surface; when treated with 5 M LiCl, the adhesion forces decreased significantly. A force–distance curve of *Lactobacillus* with broad minima indicates the release of the AFM tip from the surface proceeds via multiple unbinding events, whereas a force–distance curve with a sharp minimum strongly suggests that the separation of the bacterial surface and the AFM tip proceeds via a single unbinding event (Rief and Gaub, 1997). Accordingly, we conclude that the outer layer of this

bacterium is fully covered by a compact protein layer (Schär-Zammaretti and Ubbink, 2003).

CONCLUSIONS

Lactobacillus M5-L and Q8-L strains lack typical surface-layer proteins; rather, EF-Tu and SA appear to play some role in adherence to HT-29 cells. Both EF-Tu and SA may be regarded as key factors associated with the adhesion forces of *Lactobacillus* M5-L and Q8-L. The probiotic activity and bioactivity of the *Lactobacillus* M5-L and Q8-L surface-layer proteins warrant further investigation.

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