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Cell surface proteins play an important role in probiotic activities of *Lactobacillus reuteri*

Tejinder Pal Singh*, Ravinder Kumar Malik and Gurpreet Kaur

Abstract

Background: Eight *Lactobacillus reuteri* strains, previously isolated from breast-fed human infant feces, were selected to assess the potential contribution of their surface proteins in probiotic activity. These strains were treated with 5 M LiCl to remove their surface proteins, and their tolerance to simulated stomach-duodenum passage, cell surface characteristics, autoaggregation, adhesion, and inhibition of pathogen adhesion to Caco-2 cells were compared with untreated strains.

Results: The survival rates, autoaggregation, and adhesion abilities of the LiCl-treated *L. reuteri* strains decreased significantly ($p < 0.05$) compared to that of the untreated cells. The inhibition ability of selected *L. reuteri* strains, untreated or LiCl treated, against adherence of *Escherichia coli* 25922 and *Salmonella typhi* NCDC113 to Caco-2 was evaluated in vitro with *L. reuteri* ATCC55730 strain as a positive control. Among the selected eight strains of *L. reuteri*, LR6 showed maximum inhibition against the *E. coli* ATCC25922 and *S. typhi* NCDC113. After treatment with 5 M LiCl to remove surface protein, the inhibition activities of the lactobacilli against pathogens decreased significantly ($p < 0.05$). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis indicated that LR6 strains had several bands with molecular weight ranging from 10 to 100 KDa, and their characterization and functions need to be confirmed.

Conclusions: The results revealed that the cell surface proteins of *L. reuteri* play an important role in their survivability, adhesion, and competitive exclusion of pathogen to epithelial cells.

Keywords: *Lactobacillus reuteri*, Cell surface proteins, Adhesion, Pathogen inhibition

Background

Lactobacillus are natural inhabitant of human gut of healthy individuals, and some of these were clearly assessed for their probiotic characteristics [1–3]. The main properties for probiotic microorganisms consist in equilibrating the endogenous microflora, in protecting the gut from pathogens invasion by competitive exclusion and production of antimicrobial molecules, and in stimulating mucosal immunity. The ability to adhere to the intestinal epithelial cells is considered important in the selection of lactobacilli for probiotic use. Mechanisms of competitive exclusion of pathogens include the ability to adhere to host cells, often exerted through the same type of adhesins employed by pathogens as a strategy for gut colonization. In addition to other factors, probiotic bacterial adherence is often associated with the immunological effects of probiotic bacteria and with the interference of the adhesion of pathogenic bacteria [4].

The Gram-positive cell envelope consists of two main layers, the cytoplasmic membrane and peptidoglycan (or cell wall). Both of these layers are spanned by various proteins, such as transporters, and also, there are proteins attached on the cell surface. Several reports have appeared describing or assuming functions of cell surface proteins which includes its function as a protective sheath against hostile environmental agents, a cell shape determinant, and a sheath to mask properties of the underlying cell wall such as charge and phage receptors [5, 6]. There is also increasing evidence that bacteria may employ variation in surface proteins, by expressing alternative cell surface protein genes, for adaptation to different stress factors, such as the immune response of the host for pathogens and drastic changes in the environmental conditions for nonpathogens [6, 7]. It has been proposed that cell surface proteins are involved in cell

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protection and surface recognition and that they could be potential mediators in the initial steps involved in autoaggregation and adhesion [8–10]. In cell envelope proteome studies of potentially probiotic bacteria, the cell wall protein fraction has typically been extracted by lysozyme-containing buffer [11–16], lithium chloride [16, 17], or some other cell surface molecule or protein-solubilizing agent [15–18].

In our efforts to study the importance of cell surface proteins in probiotic activity of *Lactobacillus reuteri* strains, recovered from breast-fed human infant feces [19], our data demonstrates that cell surface proteins of *L. reuteri* play an important role in survivability, adhesion, and competitive exclusion of pathogen to epithelial cells.

Methods

Bacterial strains and growth conditions

L. reuteri LR5, LR6, LR9, LR11, LR19, LR20, LR26, and LR34 were the laboratory strains, recovered from the breast-fed human infant feces, selected for this study. The reference strain of *L. reuteri* ATCC55730 was obtained from Biogaia, Sweden. All the *L. reuteri* strains were grown in MRS broth (deMan, Ragosa, and Sharp broth; Himedia, Mumbai, India) at 37 °C for 18–24 h and maintained as glycerol stocks until further use. From these stocks cultures, working cultures were prepared and propagated twice prior to use by subculturing in MRS broth.

Removal of cell surface proteins

To remove the cell surface proteins, the bacterial cells were collected by centrifugation at 5000*g* for 15 min followed by washing with sterile distilled water and then incubating the cells in 5 M LiCl for 30 min.

Survival in simulated stomach and duodenum passage

This assay represents a simplified and standardized test system giving predictive values for the assumed survival of lactobacilli in the human stomach and duodenum under "normal" conditions [19]. The principle involves first a simulation of the stomach containing ingested lactobacilli after a meal. After 1 h, bile and artificial duodenal secretions are added in order to simulate the further passage. The MRS broth was prepared following the manufacturer's instructions, the pH adjusted to 3.0 with 5 M HCl, and then it was dispersed in the flasks containing the required volume for the test setup, followed by sterilization at 121 °C for 15 min. Synthetic duodenal juice was prepared by completely dissolving $NaHCO_3$ (6.4 g/L), KCl (0.239 g/L), and NaCl (1.28 g/L) in distilled water. The pH was adjusted to 7.4 with 5 M HCl before sterilizing at 121 °C for 15 min. The oxgal solution was prepared by reconstituting 10 g of oxgal in 100 mL water and sterilizing at 121 °C for 15 min. The required volumes of the overnight cultures and MRS broth adjusted to pH 3.0 were aseptically mixed in sterile flasks to give a final concentration of 10^8 cfu/mL in MRS, and the counts were determined by spread plating. Samples were withdrawn after 1 h of incubation at 37 °C and viable counts were determined. Four milliliters of oxgal solution was added to the culture in the flasks, followed by 17 mL of duodenal juice. After mixing, the flasks were further incubated at 37 °C. Samples were withdrawn after 2 and 3 h and counts established as described above. Three independent experiments were carried out in duplicate for all the *L. reuteri* strains before and after LiCl treatment.

Determination of bacterial hydrophobicity

Microbial adhesion to solvents (MATS) was measured according to the method of Kos et al. [20]. Three different solvents were tested in this study: *n*-hexadecane (Himedia, Mumbai, India), which is an apolar solvent; chloroform (Himedia, Mumbai, India), a monopolar and acidic solvent; and ethyl acetate (Himedia, Mumbai, India), a monopolar and basic solvent. Only bacterial adhesion to *n*-hexadecane reflects cell surface hydrophobicity or hydrophilicity. The values of MATS obtained with the two other solvents, chloroform and ethyl acetate, were regarded as a measure of electron donor (basic) and electron acceptor (acidic) characteristics of bacteria, respectively [21].

Bacteria were harvested in the stationary phase by centrifugation at 5000 *g* for 15 min, washed twice, and resuspended in 0.1 mol/L KNO_3 (pH 6.2) to approximately 10^8 CFU/ml. The absorbance of the cell suspension was measured at 600 nm (A_0). One milliliter of solvent was added to 3 ml of cell suspension. After a 10-min preincubation at room temperature, the two-phase system was mixed by vortexing for 2 min. The aqueous phase was removed after 20 min of incubation at room temperature, and its absorbance at 600 nm (A_1) was measured. The percentage of bacterial adhesion to solvent was calculated as $(1 - A_1/A_0)*100$. The experiment was also carried out for *L. reuteri* strains after LiCl treatment.

Autoaggregation assay

Autoaggregation assay was performed according to Kos et al. [20] with some modifications. Bacterial cells were grown for 18 h at 37 °C in MRS broth. The cells (with and without LiCl treatment) were harvested by centrifugation at 5000 *g* for 15 min, washed twice, and resuspended in phosphate-buffered saline (PBS; pH 7.4) to give viable counts of approximately 10^8 CFU/ml. Cell suspensions (4 ml) were mixed by vortexing for 10 s and autoaggregation was determined during 5 h of incubation at room

temperature. Every hour, 0.1 ml of the upper suspension was transferred to another tube with 3.9 ml of PBS and the absorbance (A) was measured at 600 nm. The autoaggregation percentage is expressed as: $1 - (A_t/A_0)*100$, where A_t represents the absorbance at time $t = 1, 2, 3, 4$, or 5 h and A_0 the absorbance at $t = 0$.

Caco-2 cell culture and adherence assay of *L. reuteri*

Caco-2 cell culture

The Caco-2 cell line was procured from the National Center of Cell Science, Pune, India. Cells were routinely grown in Dulbecco's modified Eagle's minimal essential medium (DMEM; Sigma, USA), supplemented with 10 % fetal bovine serum (FBS; Sigma, USA), and 100 μg/ml streptomycin (Sigma, USA) and 100 U/ml penicillin (Sigma, USA) at 37 °C in a 10 % CO_2 atmosphere. For adhesion assays, Caco-2 monolayers were prepared in 6-well tissue plates. Cells were inoculated at a concentration of 7×10^4 cells per well to obtain confluence and cultured for 21 days prior to the adhesion assay. The cell culture medium was changed on alternate days, and the last two media changes were without penicillin/streptomycin.

In vitro adherence assay

Overnight cultures of lactobacilli grown in DMEM (without FBS and antibiotics) were centrifuged, washed, and re-suspended in DMEM. Viable counts were determined by plating on MRS agar. A 1.0-ml aliquot of the bacterial suspension (adjusted to 1×10^8 cfu/ml) was added to each well of the tissue culture plate and incubated in a 5 % CO_2 atmosphere. After 2 h of incubation, the Caco-2 monolayers were washed three times with sterile PBS (pH 7.4). The cells from monolayers were detached by tripsinization. One millimeter 0.25 % trypsin-EDTA solution (Sigma, USA) was added to each well, and plate was incubated for 5 min at 37 °C. The detached cells were repeatedly but gently aspirated to make homogeneous suspension. The cell suspension was then serially diluted with saline solution and plated on MRS agar. The plates were then incubated for 24–48 h at 37 °C, and colonies were counted (B_1 cfu/ml). Bacterial cells initially added to each well were also counted (B_0 cfu/ml). The adhesion percentage was then calculated as % adhesion = $(B_1/B_0)*100$. The adhesion experiment was also performed for all the *L. reuteri* strains after LiCl treatment.

Inhibition of Escherichia coli ATCC25922 and Salmonella typhi NCDC113 adherence to Caco-2 cells by L. reuteri

The inhibition ability of Lactobacillus to pathogens adherence was performed according to the previous method with some modification [22]. Eight lactobacilli as mentioned above were used. The optical density was adjusted to 1×10^8 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 surface proteins to pathogen adherence to Caco-2. *E. coli* ATCC25922 was from American Type Culture Collection (ATCC, USA) and *S. typhi* NCDC113 was from National Collection of Dairy Cultures (NCDC, India), respectively. The selected pathogens were propagated in Brain Heart Infusion broth (BHI; Himedia) and maintained as glycerol stocks.

For competition assays, 200 μl (approximately 1×10^6 cfu) of lactobacillus and 200 μl (approximately 1×10^6 cfu) of pathogens were co-cultured with Caco-2 cells in DMEM for 2 h. For exclusion assays, lactobacilli were cultured with Caco-2 cells in DMEM for 1 h. After 1 h, Caco-2 cells were washed three times with PBS (pH 7.4) and 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 0.25 % (v/v) trypsin-EDTA solution at 37 °C for 5 min and the number of viable adhering *E. coli* and *S. typhi* were determined by plating on eosin methylene blue (EMB) and Salmonella Shigella (SS) agar plates after serial dilutions, respectively. The inhibition of pathogens by lactobacillus without surface proteins was also conducted as above.

Observation by scanning and transmission electron microscopy

Aliquots of bacterial aggregates were fixed with 2.5 % (v/v) glutaraldehyde in PBS buffer. After 1 h of fixation, the cells were washed with PBS and refixed for 1 h in the dark at room temperature with PBS buffer containing 1 % osmium tetroxide. Cells were then washed three times with the same mixture and dehydrated in a concentration series (30, 50, 70, and 80 %) of ethanol solutions for 10 min each. The cells were then washed in 100 % ethanol for 10 min before being dried in a critical-point dryer (Balzers CPD 020) and coated with gold. All preparations were observed under a ZEISS EVO 18 scanning electron microscope.

To observe the surface structure of the strain, bacteria for thin section were prefixed in glutaraldehyde (3 % in phosphate buffer, pH 7.2) for 2 h at room temperature. The micrographs were taken by JEM-100CX transmission electron microscopes at an operating voltage of 100 kV.

Isolation and SDS-PAGE analysis of cell surface proteins from L. reuteri

Cell surface proteins of lactobacillus were extracted by 5 M LiCl according to the method reported by Zhang et al. [22]. *L. reuteri* LR6 showing maximum tolerance to

simulated stomach and duodenum conditions and higher adherence to Caco-2 cell lines were incubated in 30 ml MRS broth. After culturing for 18 h, cells were collected and washed twice with ice-cold sterile water. Six millimeters of 5 M LiCl was used to mix with lactobacilli. Supernatant was collected and dialyzed with PBS and then freeze dried. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed with a 5 % (*w*/*v*) stacking gel and a 12 % (*w*/*v*) separating gel. Samples of the surface proteins were dissolved in denaturing buffer and subjected to SDS-PAGE gel. Gel was stained by Coomassie brilliant blue R-250 (Sigma, USA).

Statistical analysis

The results are expressed as the mean ± SD of three independent experiments. Statistical analysis was done by StatGraphicPlus software. Data were subjected to a one-way analysis of variance (ANOVA). Differences were considered statistically significant when $p < 0.05$.

Results

Survival in simulated stomach and duodenum passage

Among eight test strains, LR6 showed the maximum survival when exposed to simulated gastric and duodenum conditions for longer period. To evaluate the survival of the *L. reuteri* strains after removal of the cell surface proteins, all the test strains and reference culture were treated with 5 M LiCl. The survival rates of the LiCl treated *L. reuteri* strains decreased significantly ($p < 0.05$) compared to that of the untreated cells as the removal of cell surface proteins decreased the survival by two to four logs after 3 h of exposure period (Table 1).

Influence of surface properties on cell surface hydrophobicity and autoaggregation of *L. reuteri* strains

To evaluate the hydrophobic/hydrophilic and Lewis acid–base properties in the cell surfaces of *L. reuteri* strains, three solvents such as *n*-hexadecane, chloroform, and ethyl acetate were employed by using the MATS method. As listed in Table 2, a strong affinity to *n*-hexadecane and chloroform as well as a low adherence to ethyl acetate indicated the hydrophobic and basic phenotype of these strains.

The maximum autoaggregation was showed by LR6 (38.89 %) followed by LR11 (28.57 %) (Table 3). The autoaggregation of the strains decreased significantly after LiCl treatment compared to untreated strains ($p < 0.05$), indicating that cell surface proteins could be associated with the autoaggregation (Table 3). The differences in the aggregative properties of untreated and LiCl *L. reuteri* strains were also illustrated by qualitative scanning and transmission electron microscopy observations (Fig. 1). Micrographs showed the spatial arrangement of microbial aggregates and also highlighted the presence of exopolymeric substances which probably act as cement between cells (*L. reuteri* without LiCl treatment). The ultrastructure of the *L. reuteri* strains were observed by transmission electron microscopy. The changes in the cell surface after treatment with 5 M LiCl could be distinctly visible in a thin-sectioned cell.

In vitro adhesion assay to Caco-2 cells

The adhesion of *L. reuteri* strains showed a great variability depending on the strain (Table 3) and varied from 21.92 % to 52 %. Among the tested strains, the most adhesive strains were *L. reuteri* LR6 (50.62 %) and LR20 (45.58 %), while the least adhesive strain was LR5

Table 1 Survival (log10 cfu/mL) of *L. reuteri* strains (with and without LiCl treatment) under simulated stomach–duodenum passage (SSDP) conditions after 1, 2, and 3 h of incubation

Strains	0 h		1 h		2 h		3 h	
	Untreated	LiCl treated	Untreated	LiCl treated	Untreated	LiCl treated	Untreated	LiCl treated
LR5	8.16 ± 0.25^{W}	8.16 ± 0.32^{w}	$7.05 \pm 0.33^{X**}$	$5.99 \pm 0.10^{x*}$	$4.70 \pm 0.17^{Y**}$	$3.86 \pm 0.50^{y*}$	$4.49 \pm 0.29^{DZ**}$	$2.26 \pm 0.13^{Ez*}$
LR6	8.44 ± 0.14^{W}	8.44 ± 0.30^{w}	$7.26 \pm 0.23^{X**}$	$6.97 \pm 0.27^{x*}$	$6.53 \pm 0.44^{Z**}$	$3.81 \pm 0.38^{y*}$	$6.91 \pm 0.15^{AY**}$	$2.48 \pm 0.36^{BCxz*}$
LR9	8.37 ± 0.42^{W}	8.37 ± 0.45^{w}	$7.65 \pm 0.3^{X**}$	$5.55 \pm 0.19^{x*}$	$5.09 \pm 0.26^{Y**}$	$3.32 \pm 0.59^{y*}$	$4.32 \pm 0.54^{DZ**}$	$2.47 \pm 0.41^{BCDz*}$
LR11	8.44 ± 0.18^{W}	8.43 ± 0.38^{w}	$7.69 \pm 0.13^{X**}$	$5.89 \pm 0.18^{x*}$	$6.63 \pm 0.15^{Y**}$	$3.13 \pm 0.18^{y*}$	$5.09 \pm 0.42^{BCZ**}$	$2.58 \pm 0.63^{Bz*}$
LR19	8.37 ± 0.19^{W}	8.37 ± 0.20^{w}	$7.19 \pm 0.12^{X**}$	$6.83 \pm 0.16^{x*}$	$4.05 \pm 0.29^{Z**}$	$3.62 \pm 0.15^{y*}$	$4.60 \pm 0.33^{DY**}$	$2.31 \pm 0.14^{DEz*}$
LR20	8.83 ± 0.13^{W}	8.83 ± 0.32^{w}	$7.32 \pm 0.2^{X**}$	$6.71 \pm 0.27^{x*}$	$5.09 \pm 0.26^{Y**}$	$3.15 \pm 0.17^{y*}$	$4.32 \pm 0.41^{DZ**}$	$2.60 \pm 0.46^{Bz*}$
LR26	8.54 ± 0.18^{W}	8.54 ± 0.31^{w}	$7.67 \pm 0.53^{X**}$	$6.24 \pm 0.18^{x*}$	$4.74 \pm 0.27^{Y**}$	$3.81 \pm 0.57^{y*}$	$4.65 \pm 0.53^{CDY**}$	$2.39 \pm 0.12^{CDEz*}$
LR34	8.59 ± 0.16^{W}	8.59 ± 0.46^{w}	$7.63 \pm 0.37^{X**}$	$5.96 \pm 0.27^{x*}$	$6.33 \pm 0.51^{Y**}$	$3.79 \pm 0.44^{y*}$	$5.22 \pm 0.29^{BZ**}$	$2.49 \pm 0.17^{BCz*}$
L. reuteri ATCC55730	8.60 ± 0.16^{W}	8.60 ± 0.33^{w}	$7.03 \pm 0.31^{X**}$	$7.15 \pm 0.17^{x*}$	$6.69 \pm 0.23^{XY**}$	$3.84 \pm 0.13^{y*}$	$6.93 \pm 0.1^{AY**}$	$2.78 \pm 0.15^{Az*}$

Data are mean ± standard deviation of three independent experiments
$^{A, B, C, D, E}$Different symbol means statistically significant difference ($p < 0.05$) within the same column for 3 h
*,**Different symbol means statistically significant difference ($p < 0.05$) within the same row between the treatments at particular time
^{WXYZ}Different symbol means statistically significant difference ($p < 0.05$) within the same row for untreated strains at different time.
^{wxyz}Different symbol means statistically significant difference ($p < 0.05$) within the same row for LiCl treated strains at different time.

Table 2 Effect of LiCl treatment on cell surface hydrophobicity of *L. reuteri* strains

Strains	*n*-Hexadecane		Chloroform		Ethyl acetate	
	Untreated	LiCl treated	Untreated	LiCl treated	Untreated	LiCl treated
LR5	30.05 ± 0.03^{bx}	17.42 ± 0.02^{cy}	25.30 ± 0.01^{hy}	37.61 ± 0.02^{hx}	37.54 ± 0.04^{bx}	7.38 ± 0.01^{ey}
LR6	19.13 ± 0. 03^{ix}	4.66 ± 0.01^{gy}	60.18 ± 0.03^{ey}	65.36 ± 0.04^{ex}	14.38 ± 0.09^{fx}	13.79 ± 0.01^{cx}
LR9	23.54 ± 0.02^{dx}	5.21 ± 0.04^{gy}	64.14 ± 0.02^{dx}	64.42 ± 0.01^{fx}	11.93 ± 0.03^{gx}	1.72 ± 0.01^{gy}
LR11	35.97 ± 0.07^{ax}	22.74 ± 0.01^{ay}	49.35 ± 0.03^{fy}	75.08 ± 0.01^{cx}	20.69 ± 0.06^{dx}	6.23 ± 0.01^{fy}
LR19	12.02 ± 0.02^{hx}	9.17 ± 0.01^{fy}	69.02 ± 0.01^{cy}	77.84 ± 0.02^{bx}	3.36 ± 0.04^{iy}	12.09 ± 0.01^{dx}
LR20	22.07 ± 0.05^{ex}	21.53 ± 0.01^{bx}	32.86 ± 0.04^{gy}	70.18 ± 0.03^{dx}	30.02 ± 0.01^{cx}	24.36 ± 0.06^{by}
LR26	13.74 ± 0.03^{gx}	10.58 ± 0.01^{ey}	74.87 ± 0.01^{bx}	75.66 ± 0.01^{cx}	17.65 ± 0.00^{ey}	58.86 ± 0.01^{ax}
LR34	15.07 ± 0.04^{fx}	12.43 ± 0.02^{dy}	49.37 ± 0.05^{fy}	51.34 ± 0.01^{gx}	8.92 ± 0.03^{hy}	12.50 ± 0.01^{dx}
L. reuteri ATCC55730	25.04 ± 0.01^{cx}	18.12 ± 0.01^{cy}	80.45 ± 0.02^{ax}	80.29 ± 0.03^{ax}	38.52 ± 0.01^{ay}	59.08 ± 0.02^{ax}

Data are mean ± standard deviation of results from three separate experiments
^{abcdefghi}Different symbol means statistically significant difference ($p < 0.05$) within the same column
^{xy}Different symbol means statistically significant difference ($p < 0.05$) within the same row between the treatments

(21.92 %). Also, a significant ($p < 0.05$) reduction in adhesion values was observed after LiCl treatment of the strains.

Inhibition of *E. coli* ATCC25922 and *S. typhi* NCDC113 adherence to Caco-2 cells by *L. reuteri*

Inhibition of *E. coli* ATCC25922 and *S. typhi* NCDC113 adherence to Caco-2 cells by *L. reuteri* strains, with or without surface proteins, is shown in Tables 4 and 5, respectively. All the lactobacillus strains significantly inhibited the adhesion of *E. coli* ATCC25922 and *S. typhi* NCDC113 to Caco-2 cells ($p < 0.05$).

In competition assay, the inhibition activity of strains LR6, LR9, LR11, and *L. reuteri* ATCC55730 against *E. coli* ATCC25922 and *S. typhi* NCDC113 was much higher than that of LR5, LR19, LR20, LR34, and LR26. The selected *L. reuteri* LR6, LR9, LR11, and *L. reuteri* ATCC55730 inhibited 40.5, 32.5, 28, and 49 % of the adherence of *E. coli* ATCC25922 to Caco-2 cells, respectively, while among test strains, *L. reuteri* LR6 showed the highest inhibition ability against *S. typhi* NCDC113 up to 52.5 %. After the surface proteins were removed by 5 M LiCl, the inhibition activity of *L. reuteri* strains against *E. coli* ATCC25922 and *S. typhi* NCDC113 were significantly reduced ($p < 0.05$).

In exclusion assay, the strains LR5, LR6, LR9, LR20, and LR26 have higher inhibition ability against *E. coli* ATCC25922 than LR11, LR19, LR34, and *L. reuteri* ATCC55730 ($p < 0.05$), whereas the strains LR6, LR9, LR19, and LR26 have higher inhibition against *S. typhi* NCDC113 than LR5, LR11, LR20, LR34, and *L. reuteri* ATCC55730. *L. reuteri* LR6 inhibited 44 % of *E. coli* ATCC25922 adhering to the cells, higher than LR5 (32.5 %), LR9 (32.5 %), LR20 (30.5 %), and LR26 (40.5 %). For *S. typhi* NCDC113, 51, 45, 37.5, and 39 % were inhibited by LR6, LR9, LR19, and LR26, respectively, with LR6 showing the highest inhibitive ability. Without surface proteins, the inhibition activity of LR6, LR9, LR19, and LR26 were significantly reduced ($p < 0.05$) against *S. typhi* NCDC113, and the inhibition

Table 3 Comparison of autoaggregation and adhesion to Caco-2 cells of *L. reuteri* strains (with and without LiCl treatment)

Strains	Autoaggregation		Adhesion to Caco-2 cells	
	Untreated	LiCl treated	Untreated	LiCl treated
LR5	25.86 ± 0.58^{d**}	5.08 ± 0.16^{c*}	21.92 ± 0.54^{f**}	8.34 ± 0.05^{cd*}
LR6	38.89 ± 0.64^{b**}	3.33 ± 0.24^{e*}	50.62 ± 0.88^{a**}	7.75 ± 0.18^{de*}
LR9	21.05 ± 0.99^{e**}	7.69 ± 0.21^{a*}	25.64 ± 0.10^{d**}	11.92 ± 0.91^{a*}
LR11	28.57 ± 0.48^{c**}	6.52 ± 0.56^{b*}	23.50 ± 0.24^{e**}	11.33 ± 0.94^{a*}
LR19	20.00 ± 1.95^{ef**}	4.44 ± 0.59^{cd*}	23.95 ± 0.29^{e**}	8.50 ± 0.35^{c*}
LR20	19.57 ± 1.15^{f**}	4.25 ± 0.47^{d*}	45.58 ± 0.35^{b**}	8.70 ± 0.06^{c*}
LR26	21.95 ± 0.83^{e**}	7.14 ± 0.35^{ab*}	24.09 ± 3.21^{e**}	8.54 ± 0.64^{c*}
LR34	21.05 ± 1.22^{e**}	3.57 ± 0.48^{e*}	39.50 ± 0.71^{c**}	9.45 ± 0.64^{b*}
L. reuteri ATCC55730	42.67 ± 0.57^{a**}	2.50 ± 0.59^{f*}	52.00 ± 0.94^{a**}	7.36 ± 0.05^{d*}

Data are mean ± standard deviation of results from three separate experiments
*,** Indicates the differences at $p < 0.05$ level existed between the LiCl treated cells and untreated ones of the same strain
^{abcdef}Different symbol means statistically significant difference ($p < 0.05$) within the same column

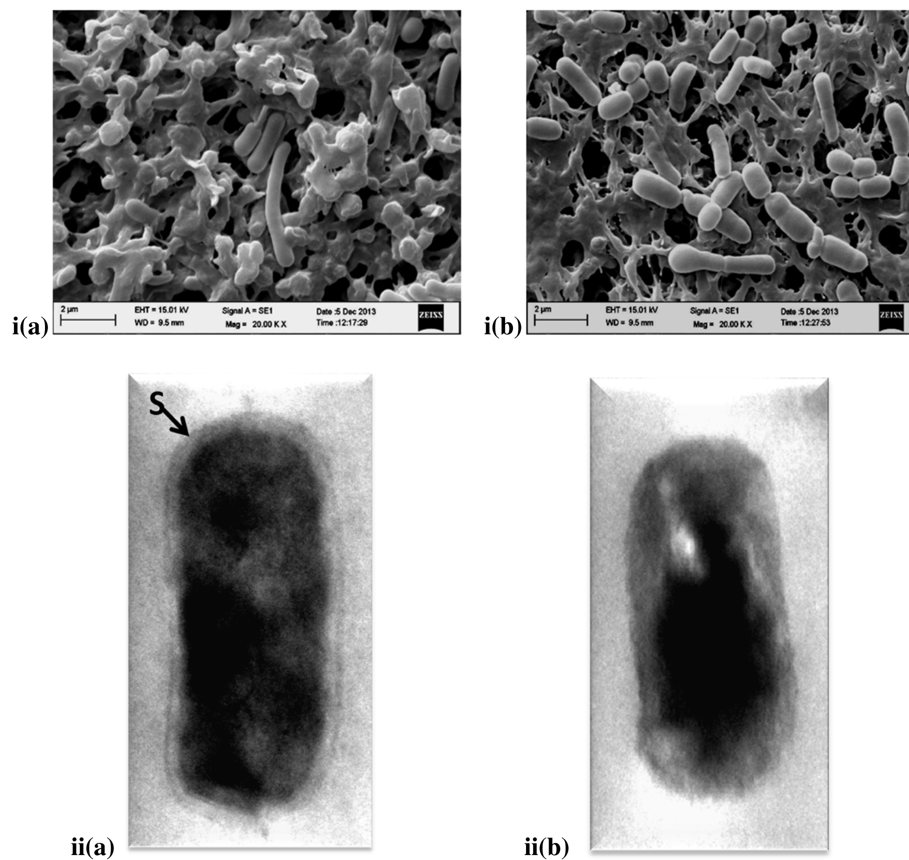

Fig. 1 **i** Examination of *L. reuteri* LR6 by scanning electron microscopy. **a** *L. reuteri* LR6 (without LiCl treatment) showing aggregation. **b** LiCl-treated *L. reuteri* LR6 showing separated cells. **ii** Examination of *L. reuteri* LR6 by transmission electron microscopy. **a** *L. reuteri* LR6 (without LiCl treatment). **b** LiCl-treated *L. reuteri* LR6. The *arrows* indicate the surface proteins of *L. reuteri* LR6

Table 4 Competition, exclusion and displacement of *E. coli* ATCC25922 adhering to Caco-2 cells by the lactobacillus strains (with or without LiCl treatment)

Strains	Competition		Exclusion		Displacement	
	Untreated	LiCl treated	Untreated	LiCl treated	Untreated	LiCl treated
LR5	85.0 ± 1.0^{bcy}	91.2 ± 1.2^{ax}	67.5 ± 1.0^{dy}	89.5 ± 1.0^{cdx}	82.5 ± 1.3^{cy}	88.2 ± 0.6^{bx}
LR6	59.5 ± 2.3^{fy}	89.4 ± 1.2^{ax}	56.0 ± 1.1^{fy}	93.1 ± 1.2^{abx}	59.5 ± 1.7^{ey}	93.2 ± 1.2^{ax}
LR9	67.5 ± 1.2^{ey}	83.7 ± 1.8^{bx}	67.5 ± 1.5^{dy}	87.4 ± 0.8^{dx}	71.5 ± 1.5^{dy}	87.3 ± 1.5^{bx}
LR11	72.0 ± 1.5^{dy}	83.9 ± 1.3^{bax}	80.5 ± 0.8^{ay}	88.4 ± 1.1^{cdx}	87.5 ± 1.7^{by}	86.0 ± 2.7^{bx}
LR19	82.5 ± 2.1^{cy}	91.3 ± 1.8^{ax}	$74.0 \pm 0.\ 4^{by}$	93.1 ± 1.3^{abx}	74.5 ± 2.1^{dy}	78.5 ± 0.7^{cx}
LR20	87.5 ± 1.7^{aby}	93.2 ± 1.5^{ax}	69.5 ± 0.9^{cdy}	90.7 ± 0.9^{bcx}	93.5 ± 1.2^{ay}	94.1 ± 0.8^{ax}
LR26	89.0 ± 1.0^{ay}	90.8 ± 2.6^{ax}	59.5 ± 1.1^{ey}	93.7 ± 1.1^{ax}	83.5 ± 2.1^{cy}	91.8 ± 0.7^{ax}
LR34	86.0 ± 1.2^{by}	92.1 ± 0.9^{ax}	81.0 ± 0.8^{ay}	92.2 ± 1.2^{abx}	73.0 ± 1.2^{dy}	92.3 ± 0.5^{ax}
L. reuteri ATCC55730	51.0 ± 0.8^{gy}	89.6 ± 2.5^{ax}	71.0 ± 0.7^{cy}	87.2 ± 1.1^{dx}	57.5 ± 1.8^{ey}	88.2 ± 0.9^{bx}

Data are adherence ratio of *E. coli* ATCC25922 to Cao-2 cells = (test/control) × 100 %, shown as mean ± standard deviation of three independent experiments
[abcdefg]Different symbol means statistically significant difference ($p < 0.05$) within the same column
[xy]Different symbol means statistically significant difference ($p < 0.05$) within the same row between the treatments

Table 5 Competition, exclusion and displacement of *S. typhi* NCDC113 adhering to Caco-2 cells by the lactobacillus (with or without LiCl treatment)

Strains	Competition		Exclusion		Displacement	
	Untreated	LiCl treated	Untreated	LiCl treated	Untreated	LiCl treated
LR5	80.0 ± 1.5^{bxy}	81.2 ± 1.2^{ax}	72.5 ± 2.1^{ay}	83.5 ± 3.6^{bcx}	75.0 ± 1.7^{cy}	80.2 ± 1.7^{cdx}
LR6	47.5 ± 1.7^{fy}	88.4 ± 1.6^{bx}	49.0 ± 2.0^{dy}	91.6 ± 1.9^{ax}	49.5 ± 2.5^{fy}	87.4 ± 1.5^{abx}
LR9	55.0 ± 0.4^{ey}	79.6 ± 2.8^{ax}	55.0 ± 1.2^{cy}	78.4 ± 2.4^{dx}	61.5 ± 1.1^{ey}	77.3 ± 2.0^{dx}
LR11	61.0 ± 0.6^{dy}	81.5 ± 1.3^{ax}	75.0 ± 1.5^{ay}	83.4 ± 1.7^{bcx}	85.0 ± 1.7^{by}	83.6 ± 2.0^{bcx}
LR19	75.0 ± 0.2^{cy}	88.7 ± 3.0^{bx}	62.5 ± 2.5^{by}	81.3 ± 1.6^{cdx}	70.0 ± 2.1^{dy}	87.8 ± 0.9^{ax}
LR20	85.0 ± 2.0^{ay}	92.3 ± 2.8^{bx}	77.0 ± 2.2^{ay}	90.7 ± 2.1^{ax}	90.0 ± 1.2^{ay}	90.3 ± 2.1^{ax}
LR26	75.0 ± 2.4^{cy}	89.1 ± 1.9^{bx}	61.0 ± 3.1^{by}	83.7 ± 2.0^{bcx}	75.0 ± 2.0^{cy}	89.1 ± 1.2^{ax}
LR34	80.0 ± 2.3^{by}	90.3 ± 1.3^{bx}	76.5 ± 3.0^{ay}	88.2 ± 2.1^{abx}	67.5 ± 2.8^{dy}	89.4 ± 1.9^{ax}
L. reuteri ATCC55730	45.0 ± 2.5^{fy}	82.6 ± 2.3^{ax}	73.0 ± 2.2^{ay}	81.7 ± 1.2^{cdx}	48.5 ± 1.8^{fy}	80.2 ± 2.1^{cdx}

Data are adherence ratio of *E. coli* ATCC25922 to Cao-2 cells = (test/control) × 100 %, shown as mean ± standard deviation of three independent experiments
^{abcdef}Different symbol means statistically significant difference ($p < 0.05$) within the same column
^{xy}Different symbol means statistically significant difference ($p < 0.05$) within the same row between the treatments

activity of LR5, LR6, LR9, LR20, and LR26 were also significantly reduced against *E. coli* ATCC25922 ($p < 0.05$).

In displacement assay, LR6 and *L. reuteri* ATCC55730 inhibited 50.5 and 51.5 % of *S. typhi* NCDC113 to adhere to Caco-2, respectively. Also, LR6 and *L. reuteri* ATCC55730 showed the highest inhibition ability to decrease 40.5 % of the *E. coli* ATCC25922, respectively. The inhibition activity of strains LR6 and *L. reuteri* ATCC55730 against *S. typhi* NCDC113 and *E. coli* ATCC25922 was significantly reduced ($p < 0.05$) on treatment with 5 M LiCl.

SDS-PAGE analysis of surface proteins

Cell surface proteins from *L. reuteri* LR6, showing maximum survival in simulated gastrointestinal conditions and highest adhesion to Caco-2 cells, were extracted with 5 M LiCl and separated on SDS gel showed bands ranging 10 to 100 kDa, as shown in Fig. 2.

Discussion

This study demonstrates the importance of cell surface proteins in probiotic activities of *L. reuteri* strains including survival in simulated gastrointestinal conditions, cell surface characteristics, aggregation properties, and adhesion abilities of selected probiotic strains and inhibition of selected pathogens to Caco-2 cells.

The survival of probiotic bacteria in the gastrointestinal ecosystem as well as adhesion to the intestinal mucosa is regarded as a prerequisite for transient colonization, stimulation of the immune system, and for antagonistic activity to enteropathogens [23, 24]. The hostile gastrointestinal conditions is the first hurdle that probiotic has to face on ingestion. The high acidic environment of stomach and high bile salts secretions in duodenum are not suitable for the survival of the bacteria. Therefore, the probiotic must be able to resist these harsh conditions. In the present study, LR6 showed maximum resistance to such unsuitable conditions. Several reports suggest that cell surface proteins act as a protective sheath against hostile environmental agents such as acid and bile salts [7]. Our study revealed that the survival of the *L. reut*eri strains significantly reduced

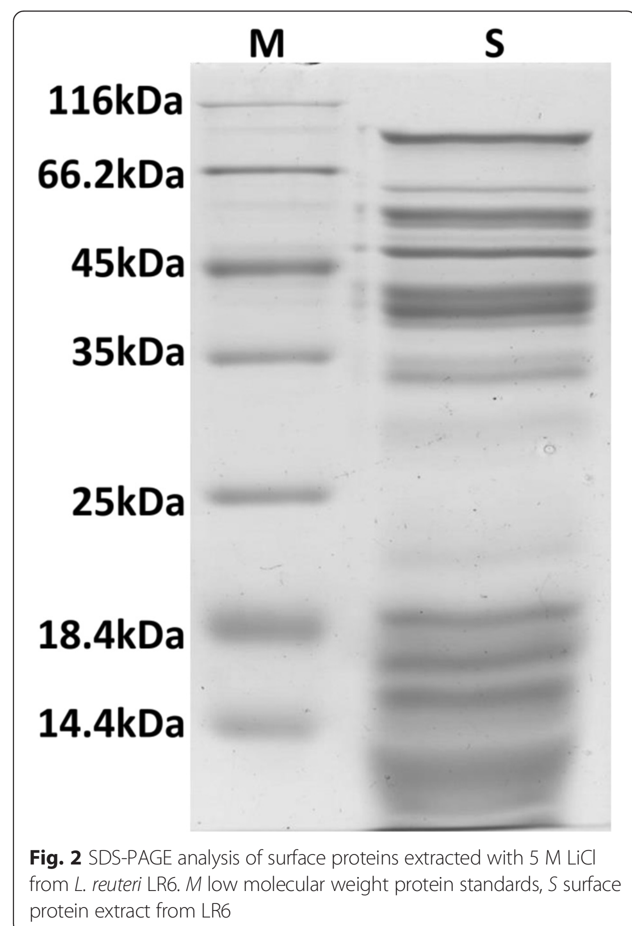

Fig. 2 SDS-PAGE analysis of surface proteins extracted with 5 M LiCl from *L. reuteri* LR6. *M* low molecular weight protein standards, *S* surface protein extract from LR6

on the removal of cell surface proteins with 5 M LiCl, confirming the protective role of their surface proteins against hostile gastrointestinal conditions.

In order to gain information on the structural properties of the cell surface of *L. reuteri* that are responsible for aggregation and adhesion, its hydrophobicity/hydrophilicity was determined. *n*-Hexadecane, chloroform, and ethyl acetate were used to assess the hydrophobic/hydrophilic, electron donor (basic), and electron acceptor (acidic) characteristics of bacterial surface, respectively (Table 2), which are attributed to carboxylic groups and Lewis acid–base interactions [20, 21]. *L. reuteri* LR11 showed higher hydrophobicity, while LR19 and LR26 showed lower hydrophobicity. The hydrophobic differences between probiotics may result in variability in their colonizing ability. Many previous studies on the physicochemistry of microbial cell surfaces have shown that the presence of (glyco-)proteinaceous material at the cell surface results in higher hydrophobicity, whereas hydrophilic surfaces are associated with the presence of polysaccharides [9, 25, 26]. The bacterial affinities to ethyl acetate were relatively low when compared to *n*-hexadecane and chloroform, indicating that probiotic strains have the nonacidic and poor electron acceptor property [25]. The autoaggregation of the probiotics varied between strains (Table 2), where *L. reuteri* LR6 showed strongest auto-aggregation ability suggesting specific binding capabilities of probiotics in the gastrointestinal tract. In most cases, autoaggregation ability was also related to cell adherence properties.

Adhesion of lactobacillus strains to the enterocyte-like Caco-2 cell model is commonly used to investigate the adhesion, inhibition, displacement, and competitive inhibition because the adhesion ability to epithelial cells is primarily considered a functional criterion for the selection of potential probiotic strains [9]. The strains with high adhesion ability can efficiently occupy the adhesive sites on the intestinal cells and mucus to inhibit the adhesion of pathogens and protect the host cells from infections. *L. reuteri* LR6 and *L. reuteri* ATCC55730 strongly adhered to Caco-2 cells (Table 3) and effectively inhibited the adherence of pathogens to Caco-2 cells (Tables 4 and 5). The observation suggests that in vitro adhesion to Caco-2 cells is correlated with competitive inhibition, which is competitively excluding entropathogens. Bacterial adhesion to the gastrointestinal tract is a complex mechanism that involves extracellular and cell surface receptors [9, 20]. To assess the potential contribution of these proteins to autoaggregation and adherence, bacterial cells were extracted with 5 M LiCl to remove surface proteins. The results showed that these proteins are important for autoaggregation in *L. reuteri* strains.

The inhibition of adhesion of different pathogens was specifically depending on the strains and pathogens used as well as the methods of assessment [27, 28]. *L. reuteri* LR6 and the reference strain *L. reuteri* ATCC55730 showed higher inhibition efficiency against *E. coli* ATCC25922 and *S. typhi* NCDC113. Other strains with high adhesive ability did not show the same inhibition capacity against *E. coli* ATCC25922 and *S. typhi* NCDC113, 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 enterotoxigenic and enteropathogenic *E. coli* [29]. *L. reuteri* LR6 and reference strain *L. reuteri* ATCC55730 with high adhesion ability generally showed much higher inhibition ability to the adherence of pathogens to Caco-2 cells, indicating that the inhibition capacity of lactobacillus against pathogenic bacteria may be related to the adhesion ability. In contrast, Collado et al. [30] found that some commercial strains with low adhesive ability 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.

For some lactobacillus strains, surface proteins perform as adhesion medium binding lactobacillus to the intestinal epithelial cells and mucus, such as mucus-binding proteins MapA from *L. reuteri* [31] and surface protein from *L. plantarum* 423 [17]. Surface 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 [32–34], have been shown to confer tissue adherence. After the lactobacillus pretreated with LiCl to remove cell surface protein, the inhibition ability of lactobacillus against pathogens decreased [27, 35–37]. In the present study, the inhibition capacity of the *L. reuteri* strains against *E. coli* ATCC25922 and *S. typhi* NCDC113 was significantly reduced when they were treated with 5 M LiCl ($p < 0.05$).

The SDS-PAGE of cell surface proteins of *L. reuteri* LR6 revealed the presence of several bands with molecular weight ranging from 10 to 100 KDa. It has been proposed that cell surface proteins are involved in cell protection and inhibition of pathogen adhesion, and they could be potential mediators in the initial steps involved in adhesion [8–10]. Recently, researchers have reported that the surface proteins from *Lactobacillus kefir* strains remained associated with *S. enteritidis* 50335 surface and could either modify or mask Salmonella structures necessary for the invasion of cultured human enterocytes instead of a competition for binding sites on the surface of the enterocyte [38]. On the other hand, surface proteins from *L. kefir* interact with the binding sites on host cell to inhibit the adhesion of *E. coli* K88 [39]. Therefore, the role of surface proteins may differ in the inhibition against pathogens. Further research is needed

to explain the adhesion mechanism as the adhesins of lactobacillus strains and the receptors expressed on host involved in adhesion are still unclear.

Conclusions

L. reuteri LR6 can be exploited as a gastrointestinal probiotics because of its resistance to acidic condition and bile salt as well as its high adhesive ability. Our findings also indicate that the cell surface proteins contributed to its increased adhesion to the cultured cells and competitive exclusion of pathogens.

Acknowledgements
We thank Dr. Sudhir Kumar Tomar, Dairy Microbiology Division, NDRI for providing the facility to carry out scanning electron microscopy.

Authors' contributions
Tejinder and Gurpreet have made substantial contributions to the conception and design, acquisition of data, and analysis and interpretation of data. The article was written by Tejinder with assistance from RK Malik and Gurpreet, taking into account the comments and suggestions of the coauthors. All coauthors had the opportunity to comment on the analysis and interpretation of the findings and approved the final version for publication.

Competing interests
The authors declare that they have no competing interests.

Received: 25 September 2015 Accepted: 16 May 2016
Published online: 28 June 2016

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