**Lactobacillus reuteri** glyceraldehyde-3-phosphate dehydrogenase functions in adhesion to intestinal epithelial cells

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**Abstract:** This study was aimed to identify key surface proteins mediating the adhesion of lactobacilli to intestinal epithelial cells. By using Caco-2 and IPEC-J2 cells labeled with sulfo-NHS-biotin in the western blotting, a protein band of an approximately 37 kDa was detected on the surface layer of *Lactobacillus reuteri* strains ZJ616, ZJ617, ZJ621, and ZJ623 and *Lactobacillus rhamnosus* GG. Mass spectrometry analysis using the adhesion-related protein from *L. reuteri* ZJ617 showed that it was 100% homologous to the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) of *L. reuteri* JCM 1112 (GenBank: YP_001841377). The ability of *L. reuteri* ZJ617 to adhere to epithelial cells decreased significantly by treatment with LiCl or by blocking with an anti-GAPDH antibody, in comparison with the untreated strain (p < 0.05). Immunoelectron microscopic and immunofluorescence analyses confirmed that GAPDH is located on the surface layer of *L. reuteri* ZJ617. The results indicated that the GAPDH protein of *L. reuteri* ZJ617 acts as an adhesion component that plays an important role in binding to the intestinal epithelial cells.

**Key words:** surface layer protein, glyceraldehyde-3-phosphate dehydrogenase, adhesion, intestinal epithelial cells, lactobacilli.

**Résumé :** La présente étude avait pour objectif d’identifier les principales protéines de surface permettant l’adhérence des lactobacilles aux cellules épithéliales intestinales. En utilisant des cellules Caco-2 et IPEC-J2 marquées à la sulfo-NHS-biotine dans l’immunobuvardage de type Western, on a détecté une bande protéique d’environ 37 kDa sur la couche de surface des souches ZJ616, ZJ617, ZJ621, ZJ623 de *Lactobacillus reuteri* et chez *Lactobacillus rhamnosus* GG. Une analyse par spectrométrie de masse de la protéine liée à l’adhérence de *L. reuteri* ZJ617 a révélé une homologie de 100 % à la glycéaldehyde-3-phosphate déshydrogénase (GAPDH) de *L. reuteri* JCM 1112 (GenBank : YP_001841377). Le pouvoir adhésif de *L. reuteri* ZJ617 à l’endroit des cellules a été significativement affaibli par un traitement au LiCl ou un blocage avec un anticorps anti-GAPDH, comparativement à la souche non traitée (p < 0,05). Des analyses de microscopie immunoélectronique et d’immunofluorescence ont confirmé que la protéine de la GAPDH de *L. reuteri* ZJ617 fait office de facteur d’adhérence et joue un rôle important dans la liaison aux cellules épithéliales intestinales. [Traduit par la Rédaction]

**Mots-clés :** protéines de la couche de surface, glycéaldehyde-3-phosphate déshydrogénase, adhérence, cellules épithéliales intestinales, lactobacilles.

**Introduction**

*Lactobacillus* strains are a group of Gram-positive bacteria that have beneficial effects on their hosts. The adhesive ability of lactobacilli to the host is often considered one of the criteria in selecting probiotic bacteria because high adhesive ability can promote the colonization of lactobacilli in the gastrointestinal tract (Edelman et al. 2012). Adhesion is a specific interaction between the surface components of lactobacilli and the host intestinal cells and mucus. Surface layer proteins (Slps) are thought to play an important role in the adherence of lactobacilli to the host. After the removal of Slps from lactobacilli, the adhesive ability of lactobacilli decreases (Frece et al. 2005; Zhang et al. 2013). The term moonlighting protein was introduced to describe the ability of proteins or peptides to have more than one function (Jeffery 2009). In addition to their conventional functions (e.g., metabolism regulation), some highly conserved cytoplasmic proteins perform moonlight activities, usually serving as an adhesin and taking place on the cell surface (Henderson and Martin 2011). In addition, aggregation promoting factors, such as elongation factor Tu (Granato et al. 2004; Ramiah et al. 2008; Muñoz-Provencio et al. 2011), the chaperonin protein complex GroEL (Bergonzelli et al. 2006; Muñoz-Provencio et al. 2011), and moonlighting protein glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Ramiah et al. 2008; Muñoz-Provencio et al. 2011), have been identified on the cell surface of lactobacilli that are involved in adhesion. LiCl treatment is often used as effective method to extract and remove Slps of bacteria. LiCl can attack Slp subunits that are noncovalently linked to bacteria while maintaining cell integrity and activity (Aguye et al. 2013). LiCl has been successfully used to remove the Slps to evaluate the autoaggregation ability of *Lactobacillus acidophilus* M92 (Kos et al. 2003) and *Lactobacillus crispatus* ZJ001 (Chen et al. 2007). Meanwhile, LiCl was successfully used to remove the Slps to evaluate the ability of *Lactobacillus kefiri* to adhere to gastrointestinal mucus (Carasi et al. 2014). In our previous study, *Lactobacillus reuteri* ZJ616, ZJ617, ZJ621, and ZJ623 and *Lactobacillus salivarius* ZJ614 isolated from piglet intestines were identified as...
strains with high adhesive ability (Zhang et al. 2013). The 5 strains showed great capacity to inhibit Escherichia coli K88 and Salmonella enteritidis 5033S from adhering to intestinal epithelial cells, and the higher adhesive ability means higher inhibition activity for lactobacilli against pathogens, in which putative Slps plays an important role (Zhang et al. 2013). In this study, we investigated the key putative Slps mediating the adhesion of these highly adhesive lactobacilli to intestinal epithelial cells.

Materials and methods

Bacterial strains and growth conditions

The 5 highly adhesive strains L. reuteri ZJ616 (GenBank accession No. JN981858), ZJ617 (JN981859), ZJ621 (JN981863), and ZJ623 (JN981865), and L. salivarius ZJ614 (JN981856) were kept in our laboratory (Zhang et al. 2013). Lactobacillus salivarius ZJ610 (JN981852) as a strain with low adhesive ability was used as the control, and Lactobacillus rhamnosus GG (LGG), which is a commercial strain with high adhesive ability (Collado et al. 2007), was selected as a positive control. Caco-2 and IPEC-J2 cells culturing and sulfo-NHS-biotin low adhesive ability was used as the control, and Lactobacillus rhamnosus GG (LGG), which is a commercial strain with high adhesive ability (Collado et al. 2007), was selected as a positive control. All of the strains were cultured in de Man–Rogosa–Sharpe broth anaerobically at 37 °C and collected at logarithmic growth phase for later use.

Caco-2 and IPEC-J2 cells culturing and sulfo-NHS-biotin labeling

The Caco-2 cell line was cultured as previously reported (Zhang et al. 2013). Caco-2 cells are commonly used as representative intestinal epithelial cells but are of cancer origin from humans. IPEC-J2 cells (nontransformed epithelial cells from a newborn pig intestinal epithelial cells but are of cancer origin from humans. E. coli K88 and S. enteritidis 5033S were used to determine the antibacterial activity against these strains.

SDS-PAGE analysis of the Slps and western blotting with sulfo-NHS-biotin-labeled cells

The putative Slps of the lactobacilli were extracted with 5 mol/L LiCl and adjusted to 10 μg of protein to be loaded in each lane (Zhang et al. 2013). The pellets of the lactobacilli treated with or without LiCl were collected and adjusted to the same concentration of an OD600 = 1 with 0.01 mol/L PBS. The blot assay was conducted as per previous reports (Chen et al. 2007a; Wang et al. 2008). The PAGE gel was transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore). The membrane was blocked, washed with PBS containing 0.05% Tween-20 (PBS-T buffer), and incubated with 100 μL of sulfo-NHS-biotin-labeled Caco-2 or IPEC-J2 cells in 10 mL of PBS-T buffer for 2 h in 10% CO2 at 37 °C. The membrane was washed and then incubated with horseradish-peroxidase-labeled streptavidin (1:400 in PBS-T buffer) (Biosynthesis Biotechnology Co., Ltd., Beijing, China) for 1 h at room temperature. The substrate 3,3-diaminobenzidine (TIANGEN, Beijing, China) was used to visualize the reactive bands.

Mass spectrometry analysis of the Slp blots with intestinal epithelial cells

The bands on the blot from the Slp of different lactobacilli strains were in the identical position; therefore, the representative band from L. reuteri ZJ617 was selected, cut out, and used for mass spectrometry analysis using a MALDI-TOF-TOF instrument (4800 proteomics analyzer; Applied Bio-systems, USA) by Shanghai Applied Protein Technology Co., Ltd. (Shanghai, China).

Expression and purification of an approximately 37 kDa protein coded by GAPDH DNA from L. reuteri ZJ617

According to the methods described previously (Hong et al. 2011) with some modifications, DNA from L. reuteri ZJ617 was extracted, and the gene coding for the GAPDH was amplified using the primers designed on the basis of the deduced amino acid sequence identified by the mass spectrometry analysis and the complete genome sequences of L. reuteri JCM 1112 (GenBank: NC_010609.1, g1|841525655:445084–446901). The primers containing the EcoRI (GAATTC) and XhoI (CTCGAG) restriction sites were as follows: forward—5’-CCGGAATTCTAGACGTTAAAATTTGATTATTT-3’; reverse—5’-CCGCTCGAGTTAAGTGAAAAATCGAAGGTAATGTTT-3’. The PCR conditions were as follows: 94 °C for 5 min for denaturation, followed by 30 cycles of 94 °C for 30 s, 55.5 °C for 1 min, and 72 °C for 1 min, and a final extension for 5 min at 72 °C. The amplified fragment was purified and ligated into the pET-30a(+) vector (Novagen, Beijing, China). The construct was transformed into E. coli BL21 (DE3) cells. Positive clones were picked and inoculated into 4 mL of Luria–Bertani (LB) medium, followed by growth at 37 °C until the OD600 reached 0.6–0.8 in 100 mL of LB medium. After the addition of IPTG (1 mmol/L), the culture was incubated for 6–8 h. The pellets were washed 3 times with PBS, suspended in 30 mL of ice-cold PBS, and broken with an Ultrasonic Cell Disruption System. The supernatant was purified by 6xHis-tagged Ni-NTA agarose (QIagen, Germany). The purified GAPDH protein was identified by SDS-PAGE and western blotting with mouse anti-6×His tag antibody. The expressed GAPDH purified protein was sent to Sangon Biotech Co., Ltd. (Shanghai, China) for generation of a rabbit polyclonal antibody.

Assay of adhesion ability of GAPDH antibody-treated ZJ617 to IPEC-J2 cells

The Lactobacillus pellets (3 × 10⁸ cfu) were washed 3 times with PBS and incubated with the anti-GAPDH antibody for 30 min at room temperature. The antibody-treated Lactobacillus strain was washed with PBS and suspended with DMEM/F-12. The assay to determine the adherence of L. reuteri ZJ617 to the IPEC-J2 cells after treatment with or without LiCl or after treatment with the antibody was conducted as previously described by Zhang et al. (2013). One millilitre of lactobacilli and 1 mL of culture medium DMEM/F-12 (Gibco, USA) were added to the cells growing in 6-well plates with glass coverslips inside, and were incubated for 2 h at 37 °C with 5% CO2. The cell monolayer was washed 3 times with PBS, fixed with 10% formaldehyde, Gram-stained, and examined by microscope. The lactobacillus adherence index was determined as per 100 IPEC-J2 cells in 20 random microscopic fields. Each assay was performed in triplicate.

The experiments of competition, exclusion, and displacement of E. coli K88 or S. enteritidis 5033S adhering to IPEC-J2 cells by L. reuteri ZJ617 were performed according to our previous report (Zhang et al. 2013). The rabbit anti-GAPDH antibody (Fig. S2) was used with some modification. For the competition assay, a total of 200 μL (approximately 1 × 10⁶ cfu) of lactobacilli and an equal amount (approximately 1 × 10⁶ cfu) of pathogens were cocultured with IPEC-J2 cells in DMEM for 2 h. For the exclusion assays, lactobacilli were cocultured with IPEC-J2 cells in DMEM for 1 h. After the IPEC-J2 cells were washed 3 times with PBS (pH 7.4), pathogens were added and incubated for 1 h. For the displacement assays, pathogens were added first and cultured for 1 h, and the lactobacilli were added subsequently and cultured for 1 h. The cells were then lysed by adding 0.05% (v/v) Triton X-100 solutions on ice for 10 min. The number of viable adhering E. coli K88 and S. enteritidis was determined by the modified MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide) colorimetric assay.
Fig. 1. Western blots of the proteins involved in adhesion to Caco-2 cells or IPEC-J2 cells and adhesive protein sequence analyzed by mass spectrometry. The surface layer proteins were extracted from the *Lactobacillus* strains ZJ610, ZJ614, ZJ616, ZJ617, ZJ621, ZJ623, and LGG with 5 mol/L LiCl. The proteins were transferred to a PVDF membrane after SDS–PAGE and western blotted with the sulfo-NHS-biotin-labeled Caco-2 cells (a) or IPEC-J2 cells (b). The reacting bands binding to the cells were visualized by a 3,3-diaminobenzidine horseradish peroxidase color development kit. ZJ610 and ZJ614, *Lactobacillus salivarius*; ZJ616, ZJ617, ZJ621, and ZJ623, *Lactobacillus reuteri*; LGG, *Lactobacillus rhamnosus* GG. The adhesive protein from ZJ617 indicated by an arrow was sent for mass spectrometry analysis, generated the basepeak profile of peptide ions (c) and the sequence (d).

(d) 1 mtvkigigf grigrlafrr ihelntndie vvaindltp smlayllkyd sthgkfpgev
   61 tstdtgivvd gkeypvyaeer darnipwvkn dgdvdfvlect gfytsaeksq ahidagakrv
   121 lisapagnip tvvgvnltd ltkddilvsaa gscctsclap mkvlndeefg vkgvtmttih
   181 aftstqaidl qprgkkmrnn rtasvntiph ssgaakaigl vipelngkls ghaqrvgvvd
   241 gsltelvsil dkkvtvdqin damkkatnpa fgytedeivs tdiigstygsvfdpsqteim
   301 egddgsqlvkgvavwyndeyg ftnmirtll hfat1
Fig. 2. SDS–PAGE and western blot analysis of the purified GAPDH (glyceraldehyde-3-phosphate dehydrogenase) protein from recombinant Escherichia coli BL21 (DE3). (a) SDS–PAGE analysis of the purified GAPDH protein. Lanes: M, low-molecular-mass protein standard; TP, the proteins extracted from the recombinant E. coli BL21 (DE3) (total protein); PP, proteins penetrating through the Ni-NTA agarose; W1–W3, proteins washed with 20, 20, and 50 mmol/L imidazole washing buffer, respectively; E1–E4, proteins obtained with 250 mmol/L imidazole elution buffer. (b and c) Western blot analysis of the purified GAPDH protein — (b) lane 1, western blotting with mouse anti-6×His tagged IgG antibody; (c) western blotting with anti-GAPDH rabbit antibody.

50335 was determined by plating on EMB and SS agar plates, respectively, after serial dilutions.

The data of adhesive ability were analyzed in a completely randomized design using the General Linear Models procedure of SAS software (SAS Institute Inc. 1996). Duncan’s new multiple range test was applied for testing the differences among the means for the LiCl or anti-GAPDH antibody treated ZJ617.

**Indirect immunofluorescence**

The pellet of Lactobacillus strain ZJ617 was washed 3 times with PBS and fixed with formaldehyde at 4 °C. After washing 3 times, the pellet was suspended by PBS and adjusted to a concentration of $10^9$ cfu/mL. One millilitre of the suspension was centrifuged at 10000 rpm and the pellet was suspended by PBS and adjusted to a concentration of 2.5% (v/v) formaldehyde at 4 °C. After washing 3 times, the pellet was resuspended in 500 μL of PBS, spread on the glass slides, air-dried, heat-fixed, and analyzed by fluorescence microscopy.

**Immunoelectron microscopy**

The immunoelectron microscopic examination of GAPDH on L. reuteri ZJ617 was conducted according to a previous method (Li et al. 2012) using the rabbit anti-GAPDH antibody. Lactobacillus reuteri ZJ617 was washed 3 times with PBS and mixed with 0.5% (m/v) agarose agar to form a solid. The sample was fixed and dehydrated with a graded ethanol series and embedded into K4M resin. After polymerization, ultrathin sections were cut and collected onto carbon-coated 200-mesh nickel grids. The sections were incubated with a rabbit anti-GAPDH antibody and Protein A-gold (10 nm in size, Sigma). After being washed with PBS, the sections were post-stained in uranyl acetate and lead citrate before examination with transmission electron microscopy (JEM-1230, JEOL Company, Japan).

**Results**

**Identification of the Slps involved in adhesion to IPEC-J2 and Caco-2 cells**

SDS–PAGE and sulfo-NHS-biotin assays were used to identify the Slps involved in adhesion, and mass spectrometry analysis was performed to determine the protein sequence. Either the IPEC-J2 or Caco-2 cells labeled by sulfo-NHS-biotin blotted with a band of approximately 37 kDa protein from the Lactobacillus strains ZJ610, ZJ614, ZJ616, ZJ621, ZJ623, and LGG, indicating that this protein is involved in adhesion and functions as an adhesin (Fig. 1a). No band was detected in the Slps from Lactobacillus strains ZJ610 and ZJ614. When the pellets of the Lactobacillus strains were treated with or without LiCl, the protein bands of the same size were less evident on strains ZJ616, ZJ617, ZJ621, ZJ623, and LGG treated with LiCl. There was no difference between strains ZJ610 or ZJ614 (Fig. S1).

In accordance with the above results, the adhesive protein was cut out and analyzed by mass spectrometry analysis. The sequence of this protein showed 100% homology to that of the GAPDH protein of L. reuteri JCM 1112 (GenBank: YP_001843177) by BLAST search in the NCBI database (Fig. 1b). The mass of this protein was approximately 36 kDa with an isoelectric point of 5.33.

**Preparation of the GAPDH protein of L. reuteri and its antibody**

To confirm the role of the GAPDH of L. reuteri in adhesion and localization on the surface layer of lactobacilli, the GAPDH protein was expressed in vitro in an E. coli system and its antibody was prepared. A pair of primers was designed based on the published GAPDH gene sequence of L. reuteri JCM 1112. The GAPDH gene was successfully obtained by PCR using L. reuteri ZJ617 DNA as the template. The PCR product was ligated into pET-30a(+) and transformed into E. coli BL21 (DE3) cells. The protein was expressed and subsequently purified by affinity chromatography (Fig. 2a). The expressed GAPDH was identified by anti-His-tagged IgG (Fig. 2b) and was subsequently used to generate an anti-GAPDH polyclonal antibody in rabbits. The antibody showed a high affinity with the GAPDH protein expressed in E. coli (Fig. 2c).
Fig. 4. Adherence to IPEC-J2 cells of Lactobacillus reuteri ZJ617 treated with LiCl or an anti-GAPDH (glyceraldehyde-3-phosphate dehydrogenase) antibody. (a) Control, L. reuteri ZJ617 adherence to cells without any treatment. (b) Adherence of L. reuteri ZJ617 to cells after L. reuteri was treated with LiCl. (c) Adherence of L. reuteri ZJ617 to cells after L. reuteri was blocked by a GAPDH antibody. The arrows show the agglutination phenomenon of the strain. (d) Adherence of L. reuteri ZJ617 to cells after different treatments: Control, L. reuteri ZJ617; ZJ617+S, L. reuteri ZJ617 treated with LiCl; ZJ617+antibody, L. reuteri ZJ617 blocked by GAPDH antibody. Each assay was performed in triplicate. Bars with different letters are statistically significantly different (p < 0.05).

Western blot of surface proteins with a rabbit anti-GAPDH antibody

The Slps of Lactobacillus strains ZJ610, ZJ614, ZJ616, ZJ617, ZJ621, ZJ623, and LGG on the PVDF membrane were blotted with the anti-GAPDH rabbit antibody. A blot band of ~37 kDa in size was identified (Fig. 3). The band of LGG was weak compared with the other strains. No band was found for strains ZJ610 and ZJ614 after blotting (Fig. 3).

Adhesive ability of L. reuteri ZJ617 treated with LiCl or an anti-GAPDH antibody

Compared with the control strain without any treatments (p < 0.05) (Fig. 4a), the adhesive ability of L. reuteri ZJ617 decreased significantly (p < 0.05) after treatment with LiCl (Fig. 4b) or with an anti-GAPDH antibody (Fig. 4c). The number of L. reuteri ZJ617 cfu adhering to IPEC-J2 cells significantly (p < 0.05) decreased to 0.55 cfu/cell after treatment with LiCl, or to 0.56 cfu/cell after blocking by an anti-GAPDH rabbit antibody; while the value was 4.08 cfu/cell for the untreated strain (Fig. 4d). Furthermore, the role of GAPDH in inhibition of ZJ617 against E. coli and S. enteritidis was evaluated. When L. reuteri ZJ617 was treated with anti-GAPDH antibody, the inhibition ability of anti-GAPDH antibody against pathogens significantly decreased in competition and exclusion assays (p < 0.05) (Fig. S2).

Location of the GAPDH protein on the surface of L. reuteri ZJ617

To validate whether the GAPDH protein is present on the surface of L. reuteri ZJ617, indirect immunofluorescence analysis and immunoelectron microscopic analysis were used. In the indirect immunofluorescence analysis, the pellet from the ZJ617 strain was washed, fixed, and diluted to a certain concentration. After incubation with the anti-GAPDH rabbit antibody and FITC-conjugated goat anti-rabbit IgG, the lactobacilli were examined by microscope. The result showed that the GAPDH protein was present on the surface of L. reuteri ZJ617, as indicated by positive green fluorescent signal (Fig. 5b). The result showed that the black Protein A-gold, representing the distribution of the GAPDH protein, was located on the surface and intracellular layer of L. reuteri ZJ617 (Figs. 5d and 5e).

Discussion

The mechanism of adhesion of lactobacilli to the intestinal tract of animals or humans is complicated and poorly clarified. Slps of lactobacilli were confirmed as adhesins that play an important role in adherence to the epithelial cells (Johnson-Henry et al. 2007; Muscariello et al. 2013). Our previous results showed that the putative Slps from lactobacilli are involved in adhesion of lactobacilli and inhibition of pathogens to adhere to Caco-2 cell lines (Zhang et al. 2013). Hynönen et al. (2002) found that the S-layer protein SlaA of Lactobacillus brevis ATCC 8287 functions as an adhesin mediating the attachment to human intestinal epithelial cell lines and fibroectin. The surface protein Cbp contributes to the adhesion of lactobacilli to collagen and anti-adhesion activity against E. coli O157:H7 (Yadav et al. 2013). The ability of L. acidophilus M92 to adhere to mouse ileum epithelial cells was reduced after the removal of the Slps (Frece et al. 2005). Many kinds of Slps are located on the cell surface, and their functions in adhesion to intestinal epithelial cell have been reported (Johnson et al. 2013).

Most of the surface components from rodent-derived L. reuteri are predicted to be involved in epithelial adhesion and species-specific biofilm formation (Oh et al. 2010; Frese et al. 2011, 2013). The evolutionary patterns detected indicate a long-term association of L. reuteri lineages with particular vertebrate species and host-driving diversification. The results of a competition experiment in a gnotobiotic mouse model revealed that rodent isolates showed elevated ecological performance, indicating that evolution of L. reuteri lineages was adaptive (Oh et al. 2010). The distinct genome content of L. reuteri lineages reflected the niche characteristics in the gastrointestinal tracts of their respective hosts, and
inactivation of 7 out of 8 representative rodent-specific genes in *L. reuteri* 100-23 resulted in impaired ecological performance in the gut of mice (Frese et al. 2011). Experiments with monoassociated mice revealed that the ability of *L. reuteri* to form epithelial biofilms in the mouse forestomach is strictly dependent on the strain’s host origin, and 11 genes identified from *L. reuteri* 100-23 were predicted to play a role in biofilm formation (Frese et al. 2013).

Sulfo-NHS-biotin-labeled crude mucus was used to hybridize protein bands separated on the PVDF membrane to identify a 61.8 kDa novel adhesion-associated protein in *Lactobacillus* L15 (Chen et al. 2007b). A surface protein from *L. reuteri* JCM1081 was identified to adhere to porcine gastric mucin and human enterocyte-like HT-29 cells (Wang et al. 2008). An approximately 37 kDa band of *Lactobacillus* strains ZJ616, ZJ617, ZJ621, ZJ623, and LGG hybridized by sulfo-NHS-biotin-labeled IPEC-J2 or Caco-2 was identified as GAPDH by mass spectrometry analysis, indicating that the GAPDH protein on the surface of the *L. reuteri* strains likely acts in their adhesion to IPEC-J2 and Caco-2 cells.

Indirect immunofluorescence and immunoelectron microscopic methods confirmed that GAPDH is located on the surface of *L. reuteri* ZJ617, implying that GAPDH is present on the surface of lactobacilli and acts as an adhesin. After removal of the surface GAPDH and the Slps by LiCl or blocking by GAPDH antibody, the adhesive ability of *L. reuteri* ZJ617 decreased significantly, suggesting that GAPDH played an important role in adhesion. GAPDH is originally an essential glycolytic enzyme that catalyzes oxidation and phosphorylation of glyceraldehyde-3-phosphate to 1,3-biphosphoglycerate. However, GAPDH was recognized as nonclassical, extracellular moonlighting protein mediating microbe–host interactions in lactic acid bacteria (Johnson et al. 2013). Also, the functions of moonlighting proteins are independent, i.e., the inactivation of one function should not affect the second function and vice versa (Kainulainen et al. 2012). Moonlighting proteins appear to be common in bacteria, and they have been identified in lots of commensal bacteria (Kainulainen et al. 2012). GAPDH was also detected in the cytoplasm and on the cell surface of *L. crispatus* (Hurmalainen et al. 2007), *Lactobacillus plantarum* (Saad et al. 2009), and *Lactobacillus jensenii* (Spurbeck and Arvidson 2010) cell, and was released into cell-free buffer to bind plasminogen and to enhance its activation by human physiological plasminogen activators (Hurmalainen et al. 2007). The plasminogen (Plg)–plasin system is important in a wealth of physiological and pathological processes in mammals, and it is also utilized by several microbial pathogens for migration within the host and to

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**Fig. 5.** (a and b) Visualization of the GAPDH (glyceraldehyde-3-phosphate dehydrogenase) proteins on the surface of *Lactobacillus reuteri* ZJ617 by indirect immunofluorescence analysis with fluorescence microscopy using an anti-GAPDH antibody and a FITC-conjugated goat anti-rabbit IgG: (a) the control strain without GAPDH antibody treatment; (b) the strain treated with the anti-GAPDH rabbit antibody and the FITC-conjugated goat anti-rabbit IgG. (c, d, e) Visualization of the GAPDH on the cell surface of *L. reuteri* ZJ617 by immunoelectron microscopy using an anti-GAPDH antibody and Protein A-gold (10-nm-diameter particles) after the strain was embedded and ultrathin section was obtained: (c) the strain was not treated with the GAPDH antibody; (d) cross-section and (e) longitudinal sections of the strain treated with the GAPDH antibody and Protein A-gold.
fulfill nutritional demands (Myöhänen and Vaheri 2004; Castellino and Ploplis 2005; Lähteenmäki et al. 2005). Plg activators could convert Plg into plasmin, whose major biological function is to dissolve fibrin clots. The glycolytic enzymes enolase and GAPDH are cytoplasmic proteins that are also expressed on the surface of several bacteria. These surface-associated forms of bacterial enolase and GAPDH serve as Plg receptors to immobilize Plg and to enhance its activation. They also exhibit adhesion functions that may have a role in bacteria-host interactions (Chhatwal 2002; Bergmann et al. 2003; Lähteenmäki et al. 2007). Moreover, the identified moonlighting functions include adhesion to host epithelia, to extracellular matrices, and to secreted mucins, as well as the engagement of the host proteolytic plasminogen system and the modulation of host immune responses (Henderson and Martin 2011).

A previous study also showed that the adhesive ability of L. plantarum 423 was decreased after GAPDH was removed by guanidine–HCl and LiCl, and the ability to exclude and displace Closstridium sporogenes was reduced (Carasi et al. 2014). The cell surface GAPDH of L. plantarum LA318 can bind human A and B group antigens and human colonic mucin (Kinoshita et al. 2008a, 2008b). GAPDH was found to exist on the surface of Lactobacillus casei under acidic conditions (Nezhad et al. 2012). Many previous reports have shown that surface GAPDH can adhere to various biological materials. The adhesion of surface GAPDH from another L. plantarum to Caco-2 cells has been demonstrated (Glenting et al. 2013). In this study, the GAPDH protein from L. reuteri was also confirmed to have the capacity to bind to the intestinal epithelial cells, such as Caco-2 cells and IPEC-J2 cells. GAPDH was an important enzyme in the glycolytic pathway in cytoplasm. Our study revealed that surface GAPDH of L. reuteri Z617 also functions as an adhesive protein and plays an important role in excluding and displacing pathogens. The mechanism of GAPDH adherence to host cells will be investigated in future studies.

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