

The ability of *Lactobacillus* adhesin EF-Tu to interfere with pathogen adhesion

Akhilesh S. Dhanani · Sandeep B. Gaudana ·
Tamishraha Bagchi

Received: 1 October 2010/Revised: 8 December 2010/Accepted: 26 January 2011/Published online: 16 February 2011
© Springer-Verlag 2011

Abstract Probiotic bacteria play an important role in preventing widespread colonization of enteropathogens in human gastrointestinal tract. *Lactobacillus plantarum* CS24.2, isolated from child fecal sample, and *L. rhamnosus* GG, a widely accepted commercial probiotic strain, were examined in vitro for their ability to inhibit the colonization of enteropathogenic *Escherichia coli* (EPEC) and *Salmonella enterica* serovar Typhi (*S. Typhi*) on human intestinal epithelial cell line (Caco-2). Different adhesion assays such as competitive inhibition, adhesion inhibition, and displacement were carried out for the assessment of antagonistic activity of probiotic bacteria toward adhesion of enteropathogens to Caco-2 cells. Both the probiotic bacteria were able to inhibit pathogen colonization between 30–90% under different assay conditions. The gene coding for the known *Lactobacillus* adhesion factor—elongation factor Tu (EF-Tu)—was amplified from *L. plantarum* CS24.2 and cloned into *E. coli* expression vector, pET30(a). The partially purified recombinant EF-Tu was able to inhibit 50% adhesion of *L. plantarum* CS24.2 to Caco-2 cells but there was no effect on *L. rhamnosus* GG adhesion under competitive inhibition assay. To investigate the functional role of lactobacilli EF-Tu in pathogen inhibition to Caco-2 cells, competitive adhesion assay was carried out and there was significant reduction in the

adhesion of *E. coli* (35.3%) and *S. Typhi* (47.7%) to Caco-2 cells.

Keywords Probiotic · Competitive adhesion · *Lactobacillus* · EF-Tu · Caco-2

Introduction

The adherence of bacteria to the surface of intestinal mucosa is a key process for colonization and persistence in gastrointestinal tract (GIT). Therefore, adhesion is an important property for both pathogenic bacteria and bacteria belonging to normal gut microbiota [1, 2]. Several diseases of GIT are caused by *Salmonella enterica* serotype Typhimurium and *Escherichia coli* [3, 4]. To cause infection, adhesion to epithelium is essential; preventing pathogenic bacterial adhesion to the epithelial cells is an effective strategy to reduce pathogen-associated illness. The epithelium of GIT can be protected from colonization of pathogen by a number of mechanisms including antibiotic treatment. Probiotic therapy is seen as potential attractive alternative to antibiotic treatment to overcome problems such as emergence of multidrug-resistant bacteria and imbalance of resident gut microbiota [5, 6]. Several *Lactobacillus* strains are being explored as probiotic bacteria to restore and maintain normal gut microbiota as well as treatment of gastrointestinal diseases. Out of these, *L. rhamnosus* GG is the best studied and widely accepted standard probiotic strain [7]. This strain that has originated from the intestinal tract of a healthy human is being studied for the treatment of acute diarrhea and prevention of inflammatory bowel diseases [8, 9].

Adherence of different probiotic bacteria has been studied using various eukaryotic cell lines of human origin

Electronic supplementary material The online version of this article (doi:10.1007/s00217-011-1443-7) contains supplementary material, which is available to authorized users.

A. S. Dhanani · S. B. Gaudana · T. Bagchi (✉)
Department of Microbiology and Biotechnology Centre,
Faculty of Science, The M.S. University of Baroda,
Vadodara, Gujarat 390-002, India
e-mail: mailforbagchi@yahoo.com

such as Caco-2 and HT-29 [10–12]. Using these cell lines, several *Lactobacillus* strains have been shown to reduce the colonization of pathogenic bacteria through competition for adherence. It is believed while inhibition of pathogens is through soluble effectors; competitive exclusion of pathogenic bacteria is through direct competition for common attachment sites on gut epithelium [13–15]. The adhesive mechanism of pathogenic bacteria is well studied; however, the knowledge about surface molecules mediating the adhesion of lactobacilli to the intestinal epithelium is scant, as only few of them have been identified and characterized. The adhesion proteins that have been characterized from different *Lactobacillus* strains include mucus-binding protein (Mub/CyuL; *L. acidophilus* NCFM), mucus adhesion-promoting protein (MapA; *L. reuteri* 104R), surface layer proteins (Slp; *L. helveticus* R0052), and elongation factor Tu (EF-Tu; *L. johnsonii* NCC533) [16–19]. EF-Tu is a G-protein that facilitates the transfer of aminoacyl-tRNA to the acceptor site of the ribosome. Despite being a cytoplasmic and an anchorless protein, EF-Tu has also been found at the surface of *L. johnsonii* NCC 533 (La1) and it mediates the colonization of the bacteria by attachment to mucus and intestinal epithelium [16]. However, the mechanism by which EF-Tu interacts with intestinal cell is not well understood. The presence of EF-Tu at the surface of pathogenic *E. coli* and periplasm of *Neisseria gonorrhoeae* also has been reported [20, 21].

The aim of the present study was to investigate the interaction of two *Lactobacillus* strains, isolate *L. plantarum* CS24.2 and standard *L. rhamnosus* GG, with pathogenic strain of *E. coli* and *Salmonella enterica* serovar Typhi (*S. Typhi*). The effect of different co-culture conditions on pathogen adhesion to intestinal epithelial cell line Caco-2 has been analyzed. The applicability of EF-Tu in preventing the adhesion of pathogenic bacteria to gut epithelium was also examined by competitive exclusion of pathogens from Caco-2 cells, using partially purified recombinant EF-Tu derived from *L. plantarum* CS24.2.

Materials and methods

Bacterial strains and culture conditions

Lactobacillus plantarum CS24.2 used in this study was isolated from human child fecal sample. The isolate *L. plantarum* CS24.2 has shown significant adhesion to intestinal epithelial cell line—19 bacteria per Caco-2 cells in 24-well tissue culture plate, tolerances toward bile and acid (pH-2) at the survival rate of 12.7 and 4.73%, respectively. The isolate has also shown broad antimicrobial activity against both Gram-positive bacteria

(*Staphylococcus aureus*) and Gram-negative bacteria (*Shigella dysentery*, *Pseudomonas aeruginosa*, and *Escherichia coli*) (unpublished data). The isolate has shown the ability to stimulate Caco-2 leading to an alteration in the levels of transcript for IL-8 and TGF- β , hence demonstrating its immunomodulatory potential. The probiotic properties analyzed are comparable with other isolates and standard strains previously reported [22]. The probiotic strain *L. rhamnosus* GG (LGG) was obtained as a kind gift from Dr. Shira Doron, MD, Department of Medicine, Tufts–New England Medical Center, USA [8]. The enteropathogenic *E. coli* serotype O26:H11 (EPEC) and *S. Typhi* (MTCC 733; IMTECH, Chandigarh, India) were obtained from culture collection of our department. Lactobacilli were grown on de Man–Rogosa–Sharpe (MRS; Himedia, Mumbai, India) broth at 37 °C for 16–18 h before study. *E. coli* and *S. Typhi* were grown aerobically at 37 °C on Luria broth (Himedia) for 16–18 h.

Caco-2 cell culture

The Caco-2 cell line was obtained from National Centre for Cell Science (NCCS), Pune, India, and was routinely cultured in Dulbecco's modified Eagle's minimal essential medium (DMEM; Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% (v/v) fetal bovine serum (FBS; Sigma-Aldrich), 10 mM nonessential amino acids, 1 mM Na pyruvate, and 50 μ g/mL gentamycin, at 37 °C temperature in a humidified atmosphere containing 5% CO₂–95% air atmosphere. The media lacked gentamycin whenever antibiotic-free medium was used. To obtain monolayers for adhesion assays, the Caco-2 cells were seeded at a density of 10⁴ cells/well in 24-well standard tissue culture plates (Corning Incorporated, NY, USA) and maintained for 2 weeks after confluence. The culture medium was replaced after every 2 days.

In vitro adhesion assays

For adhesion assays, postconfluence Caco-2 cells were preincubated with antibiotic-free medium for 4 h. The pH of the media used for adhesion assay was adjusted to 6.5 before use with 1 N HCl. Lactobacilli and pathogenic strains were harvested by centrifugation (10,000g, 2 min, and 4 °C) and washed twice with Dulbecco's phosphate-buffered saline (PBS), pH 7.0 (Sigma-Aldrich), and the cell density was adjusted by measuring absorbance at 600 nm to get 1 \times 10⁸ cfu/well in 50 μ L cell suspension for each culture.

For each combination of *Lactobacillus* with enteropathogens, three competition conditions were tested: competitive inhibition, adhesion inhibition, and displacements. In competitive adhesion assay, lactobacilli and

pathogenic strains were added to the wells with Caco-2 monolayers in same number at the same time and co-incubated for 90 min. In adhesion inhibition assay, lactobacilli were allowed to adhere to Caco-2 cells for 90 min. Un-adhered bacterial cells were then withdrawn from the wells and the Caco-2 monolayers were washed twice with 1 mL PBS each. The pathogenic strains were then added and incubated for an additional 90 min. In displacement assay, pathogenic strains were added first to wells with Caco-2 cells for 90 min followed by lactobacilli adherence after removing unbound pathogenic cells as mentioned above. At the end of each assay, Caco-2 monolayers were washed twice with 1 mL PBS each. To release adhered cells, Caco-2 cells were lysed by treatment with 0.5 mL 0.05% (v/v) Triton X-100 in PBS for 20 min at 37 °C. The Caco-2 lysate including bound bacterial cells was plated after appropriate dilution on Luria agar plate, and the enumeration was done after 24-h incubation at 37 °C. Co-incubations of Caco-2 cells with pathogenic strains alone were taken as control, and the number of bacteria adhering to Caco-2 cells was considered as 100%. It was also determined that a 30-min treatment with 0.05% (v/v) Triton X-100 in PBS at 37 °C did not affect the viability of lactobacilli and pathogenic strains (data not shown). The experiments were repeated twice in duplicates in two successive passages.

Expression of EF-Tu in *E. coli*

The EF-Tu gene was amplified from *L. plantarum* CS24.2 by colony PCR. The PCR primers F: 5' GTCAGCA TATGATGGCAAAGAACATTAT 3' and R: 5' GTA TAGGATCCGTCATCAATTTCTGAAAC 3', containing recognition sequences (underlined) for restriction enzymes *Nde*I and *Bam*HI sites, respectively, were designed using sequence of EF-Tu gene (NC_004567) from *L. plantarum* WCFS1. Amplification was performed using AccuTaq (Sigma-Aldrich) under the following conditions: incubation for 6 min at 94 °C followed by 30 cycles of 30 s at 94 °C, 30 s at 55 °C and 1 min at 72 °C, and final incubation for 10 min at 72 °C before holding at 4 °C. The expected 1.2-Kb amplicon was observed on agarose gel electrophoresis. The amplicon was cloned initially into pJET1.2 blunt-end cloning vector (Fermentas, Maryland, USA). The cloned EF-Tu gene was excised from pJET-EFTu using restriction enzymes *Nde*I and *Bam*HI and subcloned by directional cloning into expression vector pET30(a) (Novagen, EMD Biosciences, Darmstadt, Germany) giving rise to pET-EFTu. This clone was confirmed by sequencing. For expression analysis, *E. coli* BL21(DE3) transformed with pET-EFTu was inoculated with overnight-grown culture in 5 mL of Luria broth containing 1% glucose and 40 µg/mL of kanamycin and incubated at 37 °C till log phase was achieved. Likewise, *E. coli* BL-21(DE3) transformed with control vector

pET30(a) was also inoculated and used as a negative control. Isopropyl- β -D-thiogalactopyranoside (IPTG; Fermentas) was added to a final concentration of 5 mM and culture was further incubated at 37 °C and aliquots were withdrawn at different time points to check for the optimal expression of recombinant protein. Induced cells of *E. coli* BL-21(DE3) transformed with pET-EF-Tu and pET30(a) were harvested by centrifugation at 10,000g \times 10 min, 4 °C, and pellet was resuspended in 1 \times gel loading buffer, boiled for 5 min, and analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using 10% polyacrylamide gels. Protein bands were visualized by staining the gel with Coomassie brilliant blue R-250 (Sigma-Aldrich).

Partial purification of recombinant EF-Tu

The cells were grown in 25 mL of Luria broth containing 1% glucose and 40 µg/mL of kanamycin and induced with 5 mM IPTG at log phase. They were harvested after 14-h incubation by centrifugation at 10,000g for 10 min at 4 °C. The cells were resuspended in 3 mL PBS and lysed by sonication (9.9 s ON, 9.9 s OFF, 3 min 30 s). The lysate was centrifuged at 10,000g for 10 min at 4 °C. The pellet obtained therefrom was resuspended in 3 mL PBS. EF-Tu was purified from semi-denaturing SDS-PAGE using an in-house electroelution apparatus. Briefly, postsonication resuspended pellet was incubated overnight at 37 °C with gel loading buffer without β -mercaptoethanol and thereafter resolved on 10% SDS-PAGE. The band corresponding to 50 kDa was excised from the gel and was electroeluted into a dialysis membrane by overnight transfer (50 V, 4 °C) in transfer buffer containing Tris-glycine. The eluted protein was dialyzed twice against 2.5% Triton X-100 in 50 mM Tris (pH 8.8) and twice against 50 mM Tris (pH 8.8). Subsequently, the eluted sample was run on 10% SDS-PAGE along with various concentrations of bovine serum albumin (BSA). The gel was then stained with silver nitrate and EF-Tu estimated by densitometric analysis employing AlphaEaseFC 4.0 software (data not shown).

Adhesion of *Lactobacillus* strains and enteropathogens to Caco-2 cells in the presence of recombinant EF-Tu

Postconfluent Caco-2 cells in 24-well tissue culture plate were pre-incubated with 50 µL of recombinant EF-Tu (8 µg/mL) and 900 µL of DMEM to give a final concentration of 400 ng/mL EF-Tu. The cells were incubated for 15 min at 37 °C followed by addition of 50 µL suspension of lactobacilli and pathogenic strains in respective wells to get 1 \times 10⁸ cfu/well. The electroeluted sample from the corresponding region of the SDS-PAGE gel lane bearing induced *E. coli* BL21(DE3) transformed with pET30(a) vector was taken as negative control for the effect of EF-Tu. After 90-min incubation in a 5% CO₂–95% air

atmosphere, adhered lactobacilli and pathogenic strains from respective wells were counted by plating on MRS and Luria agar plates, respectively. The experiment was carried out in duplicate in two successive passages.

Antibody-mediated adhesion inhibition of *Lactobacillus* strains

The antibody-mediated inhibition assay was performed with Caco-2 monolayers in 24-well tissue culture plate according to the method previously described [23]. Different concentrations of rabbit anti-EF-Tu polyclonal antibody (2 mg/mL)—5, 10, and 15% in DMEM—were initially used for adhesion inhibition. Based on the initial observation, 0.5 ml of DMEM containing 0 and 5% rabbit anti-EF-Tu polyclonal antibody and approximately 0.5×10^8 cfu of lactobacilli were added to Caco-2 monolayers. Caco-2 monolayers incubated with negative serum and lactobacilli were used as controls. After 90-min incubation in a 5% CO₂–95% air atmosphere, adhered lactobacilli from respective wells were counted by plating on MRS agar plate. The experiment was carried out in duplicate in two successive passages.

Statistics

Values are given as mean along with the standard deviation (SD) from two independent experiments in duplicate. Significant ANOVAs were followed by Dunnett test in the case of different adhesion assay to Caco-2 cells and compared with the respective control ($p < 0.05$). The statistical analysis was conducted using SigmaStat 3.5 software.

Results

Antagonistic effect of *Lactobacillus* strains on adhesion of *E. coli* and *S. Typhi* to Caco-2 cells

To mimic various in vivo conditions, adhesion assays were designed to include competitive adhesion, adhesion inhibition, and displacement, using probiotic *Lactobacillus* strains, *L. plantarum* CS24.2 and *L. rhamnosus* GG, and enteropathogens, *E. coli* and *S. Typhi*. The numbers of enteropathogens bound to Caco-2 monolayer under different competitive adhesion conditions are given in Fig. 1a, b for *L. plantarum* CS24.2 and in Fig. 1c, d for *L. rhamnosus* GG.

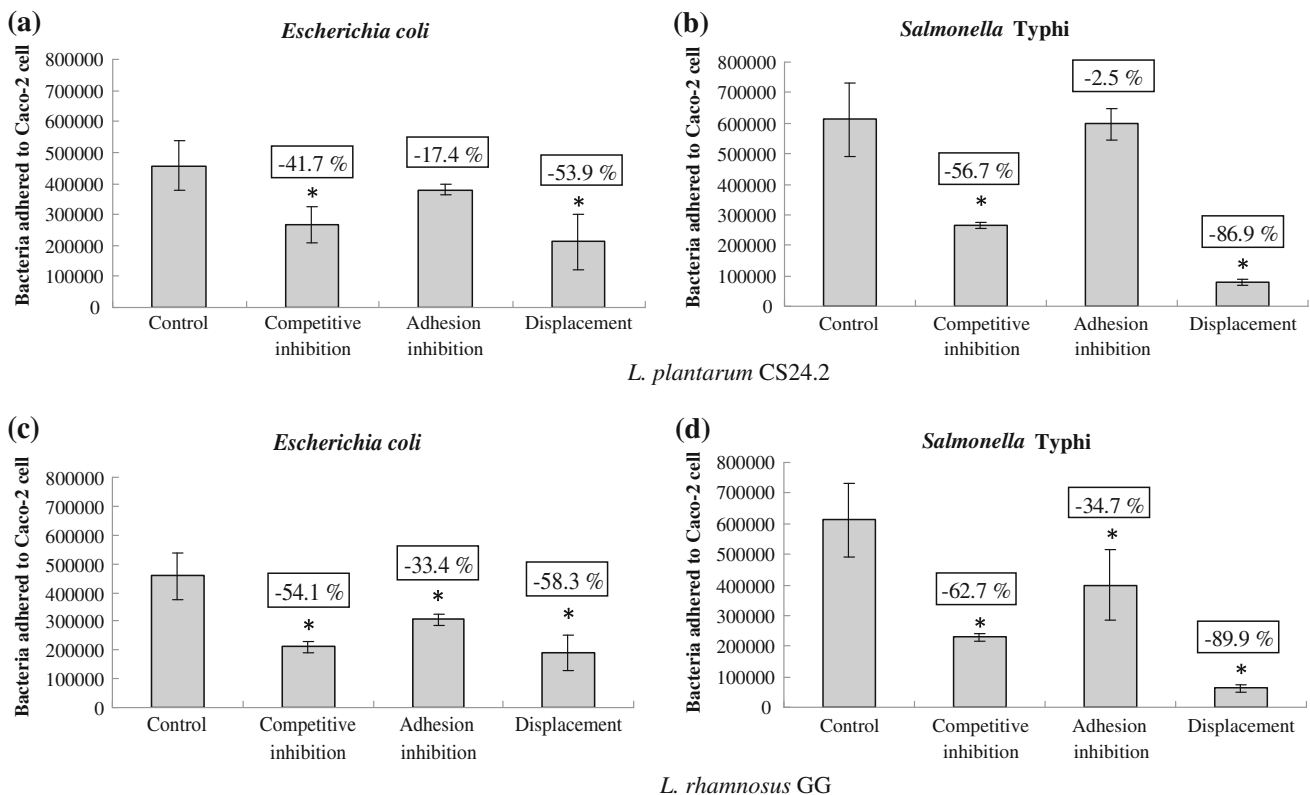


Fig. 1 Adhesion of *Escherichia coli* O26:H11 and *Salmonella Typhi* MTCC 733 to Caco-2 cells following competition with, inhibition from, and displacement by *L. plantarum* CS24.2 (a and b) and *L. rhamnosus* GG (c and d). Adhesion of *E. coli* and *S. Typhi* in the absence of lactobacilli is denoted as control. Each bar represents

mean value and standard deviation as error bar. The square box above each bar shows the percentage reduction in adhesion when compared with control. Significant ANOVAs were followed by Dunnett test for multiple comparisons versus control group. *Mean value of adhesion was significantly lower than that of control ($p < 0.05$)

In competitive adhesion assay, lactobacilli and enteropathogens were provided with a chance for binding at an equal ratio. *L. rhamnosus* competes better with both enteropathogens compared with *L. plantarum*. An adhesion inhibition assay was performed to investigate the role of lactobacilli growth and its ability to protect intestinal cells from being colonized by the pathogens. The pre-colonization of Caco-2 monolayers with *L. rhamnosus* reduced the adhesion of *E. coli* and *S. Typhi*. In contrast, no significant changes in pathogen adhesion could be detected in case of Caco-2 monolayers pre-incubated with *L. plantarum*. To investigate the ability of lactobacilli to displace colonized pathogens from intestinal epithelium, the pathogens were allowed to adhere first to Caco-2 monolayers before lactobacilli adhesion. The degree of displacement of *S. Typhi* by *Lactobacillus* strains was higher compared with that of *E. coli*.

Overall, the good adhesive strain *L. rhamnosus* GG was found to exhibit significant antagonistic activity under different adhesion assay conditions toward both pathogenic strains.

Cloning, expression, and purification of recombinant EF-Tu

The sequencing of cloned EF-Tu gene has shown 99% identity with EF-Tu gene of *L. plantarum* WCFS1. The amino acid sequence deduced from the nucleotide sequence was analyzed using Pfam database for adhesive domain search. The analysis showed that the *L. plantarum* CS24.2 EF-Tu has three adhesive domains that are an EF-Tu GTP-binding domain (PF00009), an EF-Tu domain 2 (PF03144), and an EF-Tu C-terminal domain (PF03143). Optimum time for the induction of EF-Tu in *E. coli* BL21(DE3) was observed to be 14 h (Fig. 2a). A 50-kDa band corresponding to EF-Tu was observed in the lane with *E. coli* BL21(DE3) transformed with recombinant pET-EF-Tu plasmid in contrast to that in the negative control that contained the similarly induced sample of *E. coli* BL21(DE3) transformed with control plasmid pET30(a) alone. The partially purified EF-Tu preparation was analyzed on 10% SDS-PAGE employing silver staining (Fig. 2b).

Competition assays between EF-Tu and lactobacilli for adhesion to Caco-2 cells

To evaluate the contribution of EF-Tu in adhesion of *Lactobacillus* strains to Caco-2 cells, competition assays were performed in the presence of partially purified recombinant EF-Tu preparation. In competition assays, there was significant reduction ($p < 0.05$) in adhesion of *L. plantarum* CS24.2 to Caco-2 cells, when the EF-Tu was co-incubated with the bacterial cells (Fig. 3a). Adhesion of *L. plantarum* CS24.2 was reduced by 50% in the presence of the recombinant EF-Tu

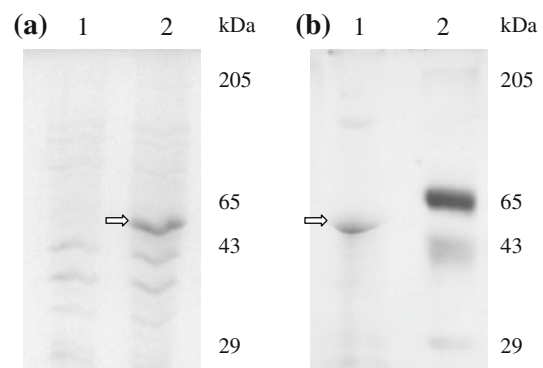


Fig. 2 **a** Coomassie-stained 10% SDS-PAGE gel with total cell lysate of *E. coli* BL21(DE3) transformed with pET30(a) (vector control; lane 1) and pET-EF-Tu (lane 2). **b** Silver-stained 10% SDS-PAGE gel with partially purified EF-Tu (lane 1) and protein marker (lane 2). Open right arrow Denotes band of 50-kDa EF-Tu

when compared with control. In contrast, there was no effect on the adhesion of *L. rhamnosus* GG when co-incubated with EF-Tu, under similar assay conditions.

Antibody-mediated inhibition assay of *Lactobacillus* strains

To further confirm the role of EF-Tu in adhesion, antibody-mediated adhesion inhibition assay was performed in the presence of rabbit anti-EF-Tu polyclonal antibody. Co-incubation with negative serum showed no effect on adhesion of lactobacilli compared with control where no serum was added. There was significant decrease ($p < 0.05$) in adhesion of *L. plantarum* CS24.2 by addition of 5% polyclonal antibody (Fig. 3b). Adhesion of *L. plantarum* CS24.2 was reduced by 22% in the presence of polyclonal antibody when compared with control. In contrast, there was no significant effect on the adhesion of *L. rhamnosus* GG when co-incubated with polyclonal antibody, under similar assay conditions.

Competition assays between EF-Tu and enteropathogens for adhesion to Caco-2 cells

To study the effect of EF-Tu on the adhesion of enteropathogens to Caco-2 cells, the bacterial cells were co-incubated with partially purified recombinant EF-Tu preparation of *L. plantarum* CS24.2 in competitive adhesion assays as described above. There was significant decrease ($p < 0.05$) in the adhesion of both enteropathogens in the presence of EF-Tu compared with control. The effect of EF-Tu addition to assay was more prominent on the adhesion of *S. Typhi* compared with that of *E. coli*. Adhesion of *E. coli* and *S. Typhi* to Caco-2 cells was strongly inhibited by addition of recombinant EF-Tu, as it was reduced by 35.3 and 47.7%, respectively (Fig. 3c).

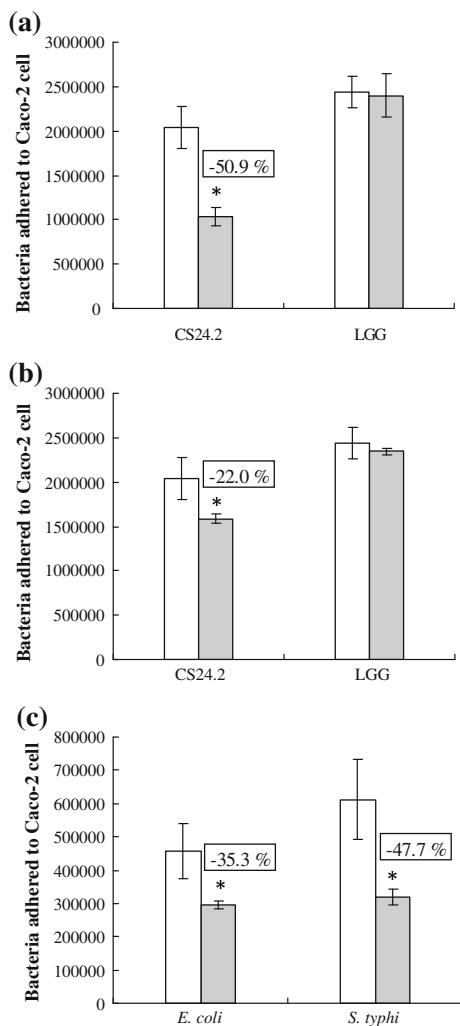


Fig. 3 **a** Adhesion of *L. plantarum* CS24.2 and *L. rhamnosus* GG to Caco-2 cells in the presence of partially purified recombinant EF-Tu (filled square box). Adhesion of lactobacilli in the absence of EF-Tu is denoted as control (open square box). **b** Effect of EF-Tu on the adhesion of *L. plantarum* CS24.2 and *L. rhamnosus* GG to Caco-2 cells determined by antibody-mediated adhesion inhibition assay (filled square box). Adhesion of lactobacilli in the absence of rabbit anti-EF-Tu polyclonal antibody is considered as absolute binding and denoted as control (open square box). **c** Adhesion of *E. coli* O26:H11 and *S. Typhi* MTCC 733 to Caco-2 cells in presence of partially purified recombinant EF-Tu (filled square box). Adhesion of *E. coli* and *S. Typhi* in the absence of EF-Tu is denoted as control (open square box). Each bar represents mean value and standard deviation as error bar. The square box above bar shows the percentage reduction in adhesion when compared with control. Significant ANOVA was followed by Dunnett test for comparison versus control group. *Mean value of adhesion was significantly lower than that of control ($p < 0.05$)

Discussion

Lactobacillus strains have been widely explored as therapeutic agents, as many of them possess health-promoting and corrective properties [24, 25]. Adhesion to intestinal epithelium is a desirable criterion for *Lactobacillus*

colonization and persistence in gastrointestinal tract before it can confer its probiotic effects to the host [26, 27]. Caco-2 cell line is widely used and accepted for study related to probiotic and pathogens adhesion to intestinal epithelium [28]. In order to study the role played by probiotic lactobacilli to protect against pathogen colonization, the competition of two *Lactobacillus* strains (*L. plantarum* CS24.2 and *L. rhamnosus* GG) and adhesive enteropathogens (*E. coli* O26:H11 and *S. Typhi* MTCC 733) for adhesion to Caco-2 cells was studied. When incubated along with pathogens, both lactobacilli showed good competitive inhibition and pathogen adhesion to Caco-2 cells decreased by around 40–60%. Lee et al. [29] under similar assay condition reported around 20–50% inhibition of strains of *E. coli* and *S. Typhimurium* adhesion to Caco-2 cells by *L. rhamnosus* GG. It is suggested that the degree of competition is strain dependent and can be determined by the affinity of adhesion molecules present on surface of respective bacteria for the receptor-binding sites that they are competing for [30].

In adhesion inhibition studies, the capacity of lactobacilli to prevent pathogen adhesion was analyzed. Only *L. rhamnosus* GG was found to exclude pathogen adhesion by around 30% when Caco-2 monolayer was pre-incubated with lactobacilli. Whether the exclusion of pathogens is due to competition for common adhesion receptors or steric hindrance of adhered lactobacilli needs to be understood. Collado et al. [31] have shown 30.3 and 27.9% adhesion inhibition of *E. coli* and *S. Typhimurium* by *L. rhamnosus* GG. Furthermore, Lee and Pong [30] have shown that the degree of adhesion inhibition depends on the relative position of the hydrophobic surface and adhesion receptors. In the present study, both lactobacilli strains examined exerted strong displacement ability toward *S. Typhi* and significant displacement toward *E. coli*. The displacement activity exerted by probiotic bacteria depends on the production of antimicrobial compounds or antiadhesion factors rather than mere competition for common adhesion receptors [32]. Candela et al. [33] showed 40–90% displacement of *E. coli* H10407 and *S. Typhimurium* from Caco-2 monolayers by strains of *L. acidophilus*. Overall, *L. rhamnosus* GG effectively reduces the adhesion of pathogens in all three adhesion assays but *L. plantarum* CS24.2 is less effective in adhesion inhibition. As per our earlier report, the adhesion ability of *L. rhamnosus* GG is higher compared with *L. plantarum* CS24.2 and, on the other hand, the antimicrobial activity is lower when compared with *L. plantarum* CS24.2 [22]. So it is indicative of the possibility that the adhesion property of *L. rhamnosus* GG may have a dominant role to play over other properties such as antimicrobial activity.

Most lactobacilli established as probiotic have been selected by their superior phenotypic properties such as adhesion to Caco-2 cells, bile and acid tolerance, and/or

antimicrobial activity [8, 34, 35]. However, studying the underlying molecular mechanisms for these properties can assist in exploring the respective molecules for direct application. A variety of adhesive molecules have been identified from different strains of lactobacilli. In case of *L. plantarum* and *L. rhamnosus* strains, few of the known adhesive molecules are alfa-enolase, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), and mucus via mannose binding (Msa) and modulator of adhesion and biofilm (MabA) and secreted LPXTG-like pilin (SpaC), respectively [36–40]. Some highly conserved cytoplasmic proteins such as EF-Tu, GAPDH, and GroEL, which lack typical signal sequence and membrane anchoring mechanism for surface expression, have been characterized [16, 37, 41]. These proteins are normally referred as anchorless multifunctional proteins or moonlighting proteins. The published genome database of the lactobacilli denotes the presence of EF-Tu gene in all those *Lactobacillus* strains which is not surprising since the elongation factor Tu (EF-Tu) is a G-protein that plays an important role in protein synthesis. Granato et al. [16] had first demonstrated the presence of EF-Tu on the surface of *L. johnsonii* NCC 533 (La1) and reported it as an adhesive molecule mediating attachment to mucin and intestinal epithelial cells.

To assess the role of EF-Tu as an adhesive molecule, we carried out heterologous expression of EF-Tu from *L. plantarum* CS24.2 into *E. coli*, followed by partial purification. Adhesion of *L. plantarum* CS24.2 to Caco-2 cells in the presence of partially purified recombinant EF-Tu was reduced by around 50%, which supports the adhesive role of EF-Tu. Similarly, Granato et al. [16] had also shown that La1 EF-Tu could prevent up to 40% adhesion of La1 bacteria to mucin. Under the present study, there was no effect on the adhesion of LGG to Caco-2 cells, which suggests that EF-Tu may not have an exclusive role in the adhesion of LGG to Caco-2. The unequivocal contribution of EF-Tu as adhesin, at least in case of *L. plantarum* CS24.2, has been reiterated by the experiment carried out using polyclonal antibody to EF-Tu. It may be interesting to note that the recently published genome sequence of LGG reveals the presence of EF-Tu (YP_003171088) and shows 95.7% similarity to La1 EF-Tu. The sequence information also suggests the presence of all 3 adhesive domains reported for La1 EF-Tu. However, it has been reported by others that in the case of fibronectin-binding protein of isogenic mutants, two lactobacilli strains with similar fibronectin-binding protein did not show similar results in adhesion to Caco-2 cells. *L. acidophilus* NCFM fbpA⁻ isogenic mutant showed reduction in adhesion to Caco-2 cells when compared with wild-type strain, while such mutation in *L. casei* did not show any reduction in adhesion to Caco-2 cells [17, 42].

Moonlighting function for many housekeeping proteins of pathogens is well documented, and many of them act as

enhancers of pathogen virulence. For example, when GAPDH is displayed on streptococcal surface, it has been reported to function as fibronectin-binding protein [43]. The mechanism of pathogen inhibition by lactobacilli is still under investigation, but Nesser et al. [44] have shown that *L. johnsonii* La1 bind to intestinal cell membrane molecules through mechanisms that are shared by pathogenic bacteria. The data presented in this study show that EF-Tu as an adhesive molecule of lactobacilli, provides beneficial effects against enteropathogen infection in vitro. The competitive adhesion analysis using recombinant EF-Tu against enteropathogens demonstrated a reduction in pathogen adhesion by 35–50%. However, it is also necessary to know whether pathogen exclusion is mediated through steric hindrance or by interacting with specific receptors. The role of EF-Tu in pathogen virulence is not yet studied but the presence of EF-Tu at the surface of pathogenic *E. coli* and membrane association is well documented [20]. Bioinformatics analysis of EF-Tu from several pathogenic strains of *E. coli* and *S. Typhi* using NCBI and Pfam databases shows around 83% similarity with La1 EF-Tu and the presence of all three adhesive domains (PF00009, PF03144, and PF03143) reported for La1 EF-Tu. So the role of these EF-Tu-like molecules in the adhesion of pathogen can be further investigated to establish EF-Tu-mediated inhibition of enteropathogens by lactobacilli.

The selection of a lactobacilli strain on the basis of its ability to antagonize specific pathogens is an important step in the development of a probiotic product for the prevention and treatment of infection caused by that pathogen. In this work, we demonstrated that probiotic strain of *L. plantarum* CS24.2 and *L. rhamnosus* GG is able to antagonize the adhesion of pathogenic strain of *E. coli* and *S. Typhi* in vitro. Earlier, Izquierdo et al. [45] demonstrated the overexpression of EF-Tu in the cell wall proteome of a highly adhesive strain *L. plantarum* WHE 92. This work further supports the role of EF-Tu in *Lactobacillus* adhesion. In conclusion, this is the first report stating that the moonlighting protein EF-Tu from probiotic lactobacilli antagonizes the adhesion of enteropathogens to intestinal cells. The study should serve as template for screening and selection of effective probiotic adjuncts as advancement in the field of food technology.

Acknowledgment The authors thank the Department of Biotechnology, New Delhi, India, for financial support (grant number BT/PR-7496/PID/20/292/2006).

References

1. Coconnier MH, Bernet MF, Kerneis S, Chauviere G, Fourniat J, Servin AL (1993) Inhibition of adhesion of enteroinvasive pathogens to human intestinal Caco-2 cells by *Lactobacillus acidophilus* strain LB decreases bacterial invasion. FEMS Microbiol Lett 110:299–305

2. Servin AL, Coconnier MH (2003) Adhesion of probiotic strains to the intestinal mucosa and interaction with pathogens. *Best Pract Res Clin Gastroenterol* 17:741–754
3. Doyle MP, Schoeni JL (1984) Survival and growth characteristics of *Escherichia coli* associated with hemorrhagic colitis. *Appl Environ Microbiol* 48:855–856
4. Rabsch W, Andrews HL, Kingsley RA, Prager R, Tschape H, Adams LG, Baumler AJ (2002) *Salmonella enterica* serotype Typhimurium and its host-adapted variants. *Infect Immun* 70:2249–2255
5. Fooks LJ, Gibson GR (2002) Probiotics as modulators of the gut flora. *Br J Nutr* 88(Suppl 1):S39–S49
6. Servin AL (2004) Antagonistic activities of lactobacilli and bifidobacteria against microbial pathogens. *FEMS Microbiol Rev* 28:405–440
7. Yan F, Polk DB (2002) Probiotic bacterium prevents cytokine-induced apoptosis in intestinal epithelial cells. *J Biol Chem* 277:50959–50965
8. Doron S, Snyderman DR, Gorbach SL (2005) *Lactobacillus GG*: bacteriology and clinical applications. *Gastroenterol Clin North Am* 34: 483–498, ix
9. Bousvaros A, Guandalini S, Baldassano RN, Botelho C, Evans J, Ferry GD, Goldin B, Hartigan L, Kugathasan S, Levy J, Murray KF, Oliva-Hemker M, Rosh JR, Tolia V, Zhouldev A, Vanderhoof JA, Hibberd PL (2005) A randomized, double-blind trial of *Lactobacillus GG* versus placebo in addition to standard maintenance therapy for children with Crohn's disease. *Inflamm Bowel Dis* 11:833–839
10. Coconnier MH, Klaenhammer TR, Kerneis S, Bernet MF, Servin AL (1992) Protein-mediated adhesion of *Lactobacillus acidophilus* BG2FO4 on human enterocyte and mucus-secreting cell lines in culture. *Appl Environ Microbiol* 58:2034–2039
11. Tuomola EM, Salminen SJ (1998) Adhesion of some probiotic and dairy *Lactobacillus* strains to Caco-2 cell cultures. *Int J Food Microbiol* 41:45–51
12. Laparra JM, Sanz Y (2009) Comparison of in vitro models to study bacterial adhesion to the intestinal epithelium. *Lett Appl Microbiol* 49:695–701
13. Coconnier MH, Lievin V, Bernet-Camard MF, Hudault S, Servin AL (1997) Antibacterial effect of the adhering human *Lactobacillus acidophilus* strain LB. *Antimicrob Agents Chemother* 41:1046–1052
14. Lehto EM, Salminen SJ (1997) Inhibition of *Salmonella typhimurium* adhesion to Caco-2 cell cultures by *Lactobacillus* strain GG spent culture supernate: only a pH effect? *FEMS Immunol Med Microbiol* 18:125–132
15. Jankowska A, Laubitz D, Antushevich H, Zabielski R, Grzesiuk E (2008) Competition of *Lactobacillus paracasei* with *Salmonella enterica* for adhesion to Caco-2 cells. *J Biomed Biotechnol* 2008:357964
16. Granato D, Bergonzelli GE, Pridmore RD, Marvin L, Rouvet M, Corthesy-Theulaz IE (2004) Cell surface-associated elongation factor Tu mediates the attachment of *Lactobacillus johnsonii* NCC533 (La1) to human intestinal cells and mucins. *Infect Immun* 72:2160–2169
17. Buck BL, Altermann E, Svingerud T, Klaenhammer TR (2005) Functional analysis of putative adhesion factors in *Lactobacillus acidophilus* NCFM. *Appl Environ Microbiol* 71:8344–8351
18. Miyoshi Y, Okada S, Uchimura T, Satoh E (2006) A mucus adhesion promoting protein, MapA, mediates the adhesion of *Lactobacillus reuteri* to Caco-2 human intestinal epithelial cells. *Biosci Biotechnol Biochem* 70:1622–1628
19. Johnson-Henry KC, Hagen KE, Gordonpour M, Tompkins TA, Sherman PM (2007) Surface-layer protein extracts from *Lactobacillus helveticus* inhibit enterohaemorrhagic *Escherichia coli* O157:H7 adhesion to epithelial cells. *Cell Microbiol* 9:356–367
20. Jacobson GR, Rosenbusch JP (1976) Abundance and membrane association of elongation factor Tu in *E. coli*. *Nature* 261:23–26
21. Porcella SF, Belland RJ, Judd RC (1996) Identification of an EF-Tu protein that is periplasm-associated and processed in *Neisseria gonorrhoeae*. *Microbiology* 142(Pt 9):2481–2489
22. Gaudana SB, Dhanani AS, Bagchi T (2010) Probiotic attributes of *Lactobacillus* strains isolated from food and human origin. *Br J Nutr* 103:1620–1628
23. Chen X, Xu J, Shuai J, Chen J, Zhang Z, Fang W (2007) The S-layer proteins of *Lactobacillus crispatus* strain ZJ001 is responsible for competitive exclusion against *Escherichia coli* O157:H7 and *Salmonella typhimurium*. *Int J Food Microbiol* 115:307–312
24. Isolauri E, Sutas Y, Kankaanpaa P, Arvilommi H, Salminen S (2001) Probiotics: effects on immunity. *Am J Clin Nutr* 73:444S–450S
25. Kim Y, Kim SH, Whang KY, Kim YJ, Oh S (2008) Inhibition of *Escherichia coli* O157:H7 attachment by interactions between lactic acid bacteria and intestinal epithelial cells. *J Microbiol Biotechnol* 18:1278–1285
26. Guarner F, Malagelada JR (2003) Gut flora in health and disease. *Lancet* 361:512–519
27. Winkler P, Ghadimi D, Schrezenmeir J, Kraehenbuhl JP (2007) Molecular and cellular basis of microflora-host interactions. *J Nutr* 137:756S–772S
28. Sambuy Y, De Angelis I, Ranaldi G, Scarino ML, Stamatii A, Zucco F (2005) The Caco-2 cell line as a model of the intestinal barrier: influence of cell and culture-related factors on Caco-2 cell functional characteristics. *Cell Biol Toxicol* 21:1–26
29. Lee YK, Puong KY, Ouwehand AC, Salminen S (2003) Displacement of bacterial pathogens from mucus and Caco-2 cell surface by lactobacilli. *J Med Microbiol* 52:925–930
30. Lee YK, Puong KY (2002) Competition for adhesion between probiotics and human gastrointestinal pathogens in the presence of carbohydrate. *Br J Nutr* 88(Suppl 1):S101–S108
31. Collado MC, Grzeskowiak L, Salminen S (2007) Probiotic strains and their combination inhibit in vitro adhesion of pathogens to pig intestinal mucosa. *Curr Microbiol* 55:260–265
32. Lievin V, Peiffer I, Hudault S, Rochat F, Brassart D, Neeser JR, Servin AL (2000) *Bifidobacterium* strains from resident infant human gastrointestinal microflora exert antimicrobial activity. *Gut* 47:646–652
33. Candela M, Perna F, Carnevali P, Vitali B, Ciati R, Gionchetti P, Rizzello F, Campieri M, Brigidi P (2008) Interaction of probiotic *Lactobacillus* and *Bifidobacterium* strains with human intestinal epithelial cells: adhesion properties, competition against enteropathogens and modulation of IL-8 production. *Int J Food Microbiol* 125:286–292
34. Goossens D, Jonkers D, Russel M, Thijs A, van den Bogaard A, Stobberingh E, Stockbrugger R (2005) Survival of the probiotic, *L. plantarum* 299v and its effects on the faecal bacterial flora, with and without gastric acid inhibition. *Dig Liver Dis* 37:44–50
35. Swetwathana A, Pilasombut K, Sethakul J (2008) Screening of bacteriocin-producing lactic acid bacteria isolated from Thai fermented meat for probiotic prospect. *J Biotechnol* 136:S737–S738
36. Castaldo C, Vastano V, Siciliano RA, Candela M, Vici M, Muscariello L, Marasco R, Sacco M (2009) Surface displaced alpha-enolase of *Lactobacillus plantarum* is a fibronectin binding protein. *Microb Cell Fact* 8:14
37. Kinoshita H, Uchida H, Kawai Y, Kawasaki T, Wakahara N, Matsuo H, Watanabe M, Kitazawa H, Ohnuma S, Miura K, Horii A, Saito T (2008) Cell surface *Lactobacillus plantarum* LA 318 glyceraldehyde-3-phosphate dehydrogenase (GAPDH) adheres to human colonic mucin. *J Appl Microbiol* 104:1667–1674

38. Kleerebezem M, Boekhorst J, van Kranenburg R, Molenaar D, Kuipers OP, Leer R, Turchini R, Peters SA, Sandbrink HM, Fiers MW, Stiekema W, Lankhorst RM, Bron PA, Hoffer SM, Groot MN, Kerkhoven R, de Vries M, Ursing B, de Vos WM, Siezen RJ (2003) Complete genome sequence of *Lactobacillus plantarum* WCFS1. Proc Natl Acad Sci U S A 100:1990–1995
39. Velez MP, Petrova MI, Lebeer S, Verhoeven TL, Claes I, Lambrichts I, Tynkkynen S, Vanderleyden J, De Keersmaecker SC (2010) Characterization of MabA, a modulator of *Lactobacillus rhamnosus* GG adhesion and biofilm formation. FEMS Immunol Med Microbiol 59:386–398
40. Kankainen M, Paulin L, Tynkkynen S, von Ossowski I, Reunanen J, Partanen P, Satokari R, Vesterlund S, Hendrickx AP, Lebeer S, De Keersmaecker SC, Vanderleyden J, Hamalainen T, Laukkanen S, Salovuori N, Ritari J, Alatalo E, Korpela R, Mattila-Sandholm T, Lassig A, Hatakka K, Kinnunen KT, Karjalainen H, Saxelin M, Laakso K, Surakka A, Palva A, Salusjarvi T, Auvinen P, de Vos WM (2009) Comparative genomic analysis of *Lactobacillus rhamnosus* GG reveals pili containing a human-mucus binding protein. Proc Natl Acad Sci U S A 106:17193–17198
41. Bergonzelli GE, Granato D, Pridmore RD, Marvin-Guy LF, Donnicola D, Cortesy-Theulaz IE (2006) GroEL of *Lactobacillus johnsonii* La1 (NCC 533) is cell surface associated: potential role in interactions with the host and the gastric pathogen *Helicobacter pylori*. Infect Immun 74:425–434
42. Munoz-Provencio D, Perez-Martinez G, Monedero V (2009) Characterization of a fibronectin-binding protein from *Lactobacillus casei* BL23. J Appl Microbiol 108:1050–1059
43. Pancholi V, Fischetti VA (1992) A major surface protein on group a streptococci is a glyceraldehyde-3-phosphate-dehydrogenase with multiple binding activity. J Exp Med 176:415–426
44. Neeser JR, Granato D, Rouvet M, Servin A, Teneberg S, Karlsson KA (2000) *Lactobacillus johnsonii* La1 shares carbohydrate-binding specificities with several enteropathogenic bacteria. Glycobiology 10:1193–1199
45. Izquierdo E, Horvatovich P, Marchioni E, Aoude-Werner D, Sanz Y, Ennahar S (2009) 2-DE and MS analysis of key proteins in the adhesion of *Lactobacillus plantarum*, a first step toward early selection of probiotics based on bacterial biomarkers. Electrophoresis 30:949–956