



***In vitro* evaluation of cell adhesion and immunomodulatory properties of five *Lactobacillus rhamnosus* strains isolated from infants**

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ABSTRACT

Aims: Five *Lactobacillus rhamnosus* strains (UBU03, UBU06, UBU09, UBU34 and UBU37) with good *in vitro* probiotic properties, isolated from breast-fed infants, were evaluated for *in vitro* adhesion, competitive adhesion and immunomodulatory properties. Knowledge of such properties is important when considering specific circumstances when these strains might be used clinically.

Methodology and results: The Caco-2 cell line was used for adhesion assays and for competitive adhesion assays against *Escherichia coli* O157:H7. *Lactobacillus rhamnosus* GG was used as the reference strain for adhesion assays. The immunomodulatory activities of the five strains were evaluated by determining the levels of the inflammatory cytokines IL-6, IL-12 and TNF- α , and of the immunoregulatory cytokine IL-10, produced by bacterial-activated THP-1 cells after 6, 12 and 24 h of stimulation. In the cell-adhesion assays, all five strains showed high adhesion properties. For UBU09, UBU34 and UBU37, adhesive capacity was higher than that of the reference strain. All strains except UBU03 showed the ability to inhibit adhesion of *E. coli* O157:H7 to Caco-2 cells. All strains induced IL-6 production but not IL-12 production. UBU03 and UBU09 could induce only one cytokine IL-6. UBU06 and UBU34 could each induce two (IL-6/IL-10 and IL-6/TNF- α , respectively). UBU37 could induce three cytokines (IL-6/TNF- α /IL-10).

Conclusion, significance and impact of study: These five probiotic *L. rhamnosus* strains with high adhesion properties and with different *in vitro* cytokine induction profiles should be investigated further in different immunological conditions to identify appropriate circumstances for their clinical use.

Keywords: Adhesion, Immunomodulation, Lactic acid bacteria, *Lactobacillus rhamnosus*, Probiotic

INTRODUCTION

Probiotics are live microorganisms that, when administered to humans in appropriate amounts, will give beneficial effects (FAO/WHO, 2002). Although strains of probiotic organisms have been isolated from various sources, most probiotics for human use have human origins (Adams, 1999; Taheri *et al.*, 2009). Two major taxa of lactic acid bacteria (LAB), i.e. lactobacilli and bifidobacteria, are the most commonly used probiotics because of their health benefits in controlling various infectious diseases. For example, *L. rhamnosus* GG strain and *Bifidobacterium bifidum* have been used for prevention and effective treatment of diarrhea caused by rotavirus (Kechagia *et al.*, 2013) and some enteric bacteria (Szajewska *et al.*, 2001). In addition, some *Lactobacillus* species have been used for effective prevention and treatment of recurrent infections in the urogenital tract, such as recurrent bacterial vaginosis (Barrons and Tassone, 2008). One important

characteristic of probiotics, besides their safety, is their ability to adhere to the host gastrointestinal (GI) tract which is required for colonization (Jensen *et al.*, 2012; Alander *et al.*, 1999), for conferring antimicrobial action (Bernet-Camard *et al.*, 1997) and for modulating the immune system (Schiffrin *et al.*, 1995). Lactobacilli can prevent the colonization of food-borne enteric pathogens such as *Escherichia coli* by competitive inhibitory adhesion to intestinal epithelial cells (Abedi *et al.*, 2013). In developing countries, one of the most common causes of morbidity and mortality is infectious diarrhea. The annual mortality rate due to this is about 4.6 to 6 million across Asia, Africa and the Americas. *Escherichia coli*, predominantly serotype O157:H7, is responsible for 30% of gastroenteritis cases (Khosravi *et al.*, 2016). Besides diarrhea, many other infectious diseases are common in developing countries. In this situation, highly desired phenotypes of probiotic LAB are those that can interfere

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with pathogens through mechanisms such as competitive adhesion, production of antimicrobial substances and/or immunomodulatory effects (Bosch *et al.*, 2012).

Little is known about the precise mechanisms by which LAB exert their immunomodulatory effects. It may be mediated via strains-specific cell-wall components, secretory metabolites or nucleic acids lead to the immune activation and subsequent effects on production of certain cytokines (Ashraf *et al.*, 2014).

The five strains of *L. rhamnosus* selected for this study had previously been isolated from breast-fed infants. These strains showed high acid- and bile-tolerance and exhibited antimicrobial activities against *E. coli* O157:H7 and/or *Vibrio cholerae* (Panya *et al.*, 2016). The five strains were evaluated for their ability to adhere to Caco-2 cells, their competitive adhesion against *E. coli* O157:H7 and their immunomodulatory properties in stimulating production of proinflammatory cytokines (IL-6, IL-12 and TNF- α) and anti-inflammatory cytokine (IL-10) from THP-1 cells. The information derived from this work could direct further study on precise mechanisms of these properties, and on proper application of these five probiotic *L. rhamnosus* strains to achieve health benefits.

MATERIALS AND METHODS

Bacterial strains and their growth conditions

Six strains of *L. rhamnosus* were used in this study; UBU03, UBU06, UBU09, UBU34, UBU37 and GG (ATCC53613). All strains were grown in Man, Rogosa and Sharpe (MRS) broth (LAB, United Kingdom) and incubated at 37 °C for 16 h in statically aerobic conditions. Agarified MRS medium was obtained by adding 15 g/L of bacteriological agar (Oxoid, USA) into MRS broth. The bacterial stock was made in skim milk solution containing 30% glycerol and stored at -80 °C until use. *Escherichia coli* O157:H7 was grown in MacConkey agar (Himedia, India) and incubated at 37 °C for 16 h.

Bacterial cell preparation

A single colony of each *L. rhamnosus* strain on an MRS agar plate was selected and grown in 10 mL MRS broth at 37 °C for 16 h in statically aerobic conditions. After incubation, the bacterial cells were harvested by centrifugation at 8000 \times g for 10 min and washed twice in phosphate buffer saline (PBS) pH7.4. *Escherichia coli* O157:H7 was grown in Luria-Bertani (LB) broth at 37 °C with shaking for 16 h before use. For any assays, the bacterial cell suspension was adjusted to McFarland no 0.5 with PBS pH 7.4 (equivalent to 1.0×10^8 CFU/mL).

Cell culture conditions

The human epithelial colorectal adenocarcinoma cell line, Caco-2, was grown in Dulbecco's Modified Eagle Medium (DMEM Glutamax, Gibco, Thermo Fisher Scientific, USA) supplemented with 10% (v/v) fetal bovine serum (FBS) (Gibco, Thermo Fisher Scientific, USA), 1% non-essential

amino acids (Gibco, Life Technologies, USA), 100 U/mL of penicillin G and 100 μ g/mL of streptomycin (Gibco, Thermo Fisher Scientific, USA).

Cells of the human monocytic leukemia line, THP-1, were grown in RPMI 1640 medium (Gibco, Thermo Fisher Scientific, USA) supplemented with 10% FBS (Gibco, Thermo Fisher Scientific, USA), 0.5 mM 2-mercaptoethanol, 100 U/mL of penicillin G and 100 μ g/mL of streptomycin (Gibco, Thermo Fisher Scientific, USA). Both Caco-2 and THP-1 cells were incubated at 37 °C with 5% CO₂ and subpassaged when the cells reached 80% confluence.

Cell adhesion assays

Cell adhesion assays were performed following Le Blay *et al.* (2004) with some modifications. The Caco-2 cell line has morphological and functional properties similar to those of the enterocytes of the small intestine, making it a suitable model for this study. Caco-2 cells were seeded at a density of 50,000 cells/cm² in each well of a 6-well plate. The experiment was performed when the cells reached 80-90% confluence and showed morphological and functional differentiation. Bacterial cells at a concentration of 1.0×10^8 CFU/mL in DMEM (without FBS and antibiotics) were transferred into each well and incubated for 1 h at 37 °C with 5% CO₂. After incubation, the plate was washed three times with 100 μ L PBS to remove non-adherent bacteria (Le Blay *et al.*, 2004). The adherent bacteria were released from Caco-2 cells by treating with 25 μ L of trypsin-EDTA solution (0.25% trypsin, 2.21 mM EDTA) (Corning, USA) for 15 min at 37 °C. After cell dissociation, ten-fold serial dilutions were made and appropriate dilutions of cell suspension were plated in duplicate on MRS agar and incubated at 37 °C for 48 h. Three independent experiments were performed for each cell adhesion assay. The percentage (%) of adherent cells was calculated as follow:

Percentage of adherent cells = $(\log CA_T / \log CA_C) \times 100$
where CA_C and CA_T represent the total viable count in CFU/mL of the tested *L. rhamnosus* strain at 0 h and 1 h of incubation, respectively.

Competitive adhesion assays

Caco-2 cells were seeded into a 24-well plate at a density of 50,000 cells/cm². Each *L. rhamnosus* test strain and *E. coli* were adjusted with 500 μ L DMEM medium (without FBS and antibiotics) to a final concentration of 10^8 CFU/mL and added to the Caco-2 cells. After 1 h incubation at 37 °C with 5% CO₂, the non-adhered bacterial cells were removed by washing three times with PBS while the adhered bacterial cells were recovered by treatment with trypsin-EDTA solution for 10 min at 37 °C. The recovered bacterial cells were plated on selective medium for counting: MRS agar for *Lactobacillus* sp. and MacConkey agar for *E. coli* O157:H7. Three independent experiments were performed for each competitive adhesion assay. The percentage of competitive inhibition was calculated as follow (Horosova *et al.*, 2006):

% of competitive adhesion = $1 - (\log \text{ no. of adherent } E. coli / \log \text{ no. of adherent } Lactobacillus \text{ sp.}) \times 100$.

Immunomodulatory activity assays

The immunomodulatory properties of the bacterial strains were evaluated by their ability to stimulate THP-1 cells to produce proinflammatory cytokines (TNF- α , IL-6, IL-12) and the anti-inflammatory cytokine IL-10. The THP-1 cells were incubated in 24-well culture plates at a final concentration of 1×10^6 cells/mL in the presence or absence of 1×10^9 CFU/mL bacterial cells at 37 °C with 5% CO₂ for 6, 12 or 24 h (Jensen *et al.*, 2015). For the positive control, 1 μ g/mL of purified lipopolysaccharide (LPS) (Sigma-Aldrich, USA) and 100 ng/mL of phorbol myristate acetate (PMA) (Sigma-Aldrich, USA) were used to stimulate THP-1 cells. Unstimulated THP-1 cells were used as the negative control for basal cytokine production. Three independent experiments were performed for each assay. The supernatants of stimulated and unstimulated cells were harvested at 6, 12 and 24 h, aliquoted and stored at -80 °C until analysis.

Cytokine assays

Enzyme-linked immuno-sorbent assays (ELISA), with an ELISA MAX™ kit (BioLegend, USA), were used, following the manufacturer’s instructions, to determine concentrations of IL-6, IL-10, IL-12 and TNF- α in the supernatants from the immunomodulation assays. Data were expressed as the mean cytokine response minus background (pg/mL) for each treatment (Ashraf *et al.*, 2014). Detection limits of the assay for each cytokine are as follows; 4 pg/mL for IL-6 and IL-12, 2 pg/mL for IL-10 and TNF- α .

Statistical analysis

Cytokine production levels by bacterial-stimulated and unstimulated THP-1 cells were compared using one-way ANOVA (SPSS software). A p-value less than 0.05 was regarded as statistically significant.

This study has been approved by the Ethics Committee of Ubon Ratchathani University, Ubon Ratchathani, Thailand (2/2556).

RESULTS

Cell adhesion assays

All five probiotic strains showed high adhesion capacity, with 79% or more of bacterial cells adhering to Caco-2 cells (Table 1). Moreover, UBU09, UBU34 and UBU37 showed higher adhesion than the reference strain GG (94%, 87% and 90% versus 84%).

Competitive adhesion assays

As all five probiotic strains showed a high capacity to adhere to Caco-2 cells, they were further evaluated for their competitive adhesion activities against the enteropathogenic bacterium, *E. coli* (EPEC) O157:H7. *Lactobacillus rhamnosus* strains UBU06, UBU09, UBU34 and UBU37 can competitively inhibit the adhesion of EPEC to Caco-2 cells with mean percentages of inhibition being 45, 43, 43 and 33, respectively. UBU03 showed no competitive adhesion against *E. coli* O157:H7 as shown in Figure 1.

Table 1: The percentage of cells of each *Lactobacillus*

LAB strains	Percentage of adhesion
<i>L. rhamnosus</i> GG	84 ± 1.26*
<i>L. rhamnosus</i> UBU03	82 ± 2.91
<i>L. rhamnosus</i> UBU06	79 ± 1.15
<i>L. rhamnosus</i> UBU09	94 ± 4.21
<i>L. rhamnosus</i> UBU34	87 ± 0.59
<i>L. rhamnosus</i> UBU37	90 ± 0.66

rhamnosus strain adhering to Caco-2 cells.

* Mean ± S.D.

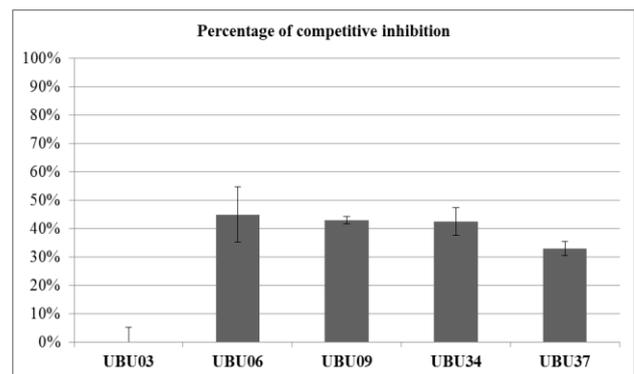


Figure 1: The competitive inhibition of five *Lactobacillus rhamnosus* strains against *E. coli* O157:H7 adhesion on Caco-2 cells. Results are presented as mean ± S.D.

Immunostimulatory assay

Levels were determined of IL-6, IL-10, IL-12p70, and TNF- α produced by THP-1 cells when stimulated by five strains of probiotic *L. rhamnosus*. As shown in Figure 2, different strains stimulated the highest level of IL-6 production at different time points. For UBU03, the highest level of IL-6 was at 6 h, whereas for the other strains the highest level of IL-6 was at 12 h. Overall, UBU03 stimulated the highest level of IL-6 production (235.5 pg/mL) from THP-1 cells. For TNF- α (see Figure 3), UBU34 and UBU37 stimulated the highest levels of production at 6 and 24 h, respectively. UBU34 stimulated the highest level of TNF- α production (192.5 pg/mL) among the five strains. UBU03, UBU06, and UBU09 stimulated THP-1 to produce TNF- α at very low and non-significant levels. For anti-

inflammatory cytokine, IL-10 (Figure 4), UBU06 and UBU37 stimulated levels of production (40.5 and 48.1 pg/mL, respectively) that were significantly higher than in the negative controls. Levels of IL-10 production stimulated by UBU03, UBU09 and UBU34 were low or undetectable. None of the five strains could stimulate THP-1 cells to produce IL-12 (data not shown). This series of experiments is summarized in Table 2. UBU37 stimulated THP-1 cells to produce significantly elevated levels of three cytokines IL-6, IL-10 and TNF- α . UBU06 and UBU34 stimulated production of two cytokines, IL-6/IL-10 and IL-6/TNF- α respectively. UBU03 and UBU09 stimulated THP-1 cells to produce IL-6 only.

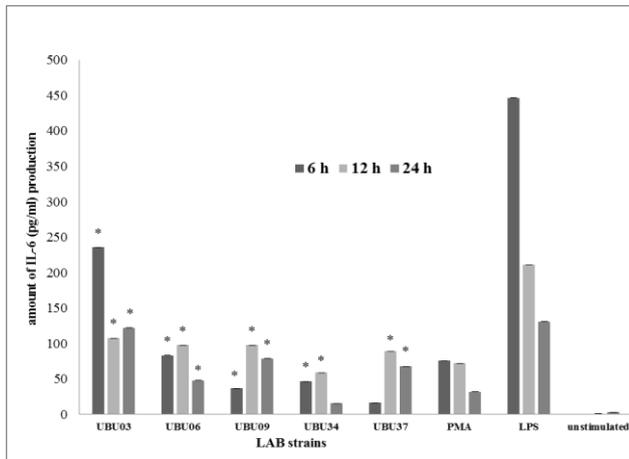


Figure 2: IL-6 production at 6 h, 12 h and 24 h from THP-1 cells after stimulation with live LAB, PMA, LPS and from unstimulated cells. Results are presented as mean \pm S.D. *p-value < 0.05 indicates statistically significant difference when comparing stimulated with unstimulated condition.

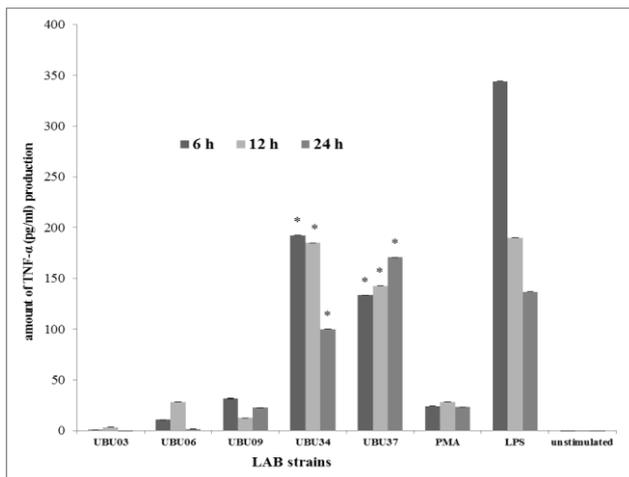


Figure 3: TNF- α production at 6 h, 12 h and 24 h from THP-1 cells after stimulation with live LAB, PMA, LPS and from unstimulated cells. Results are presented as mean \pm S.D. *p-value < 0.05 indicates statistically significant difference when comparing stimulated with unstimulated condition.

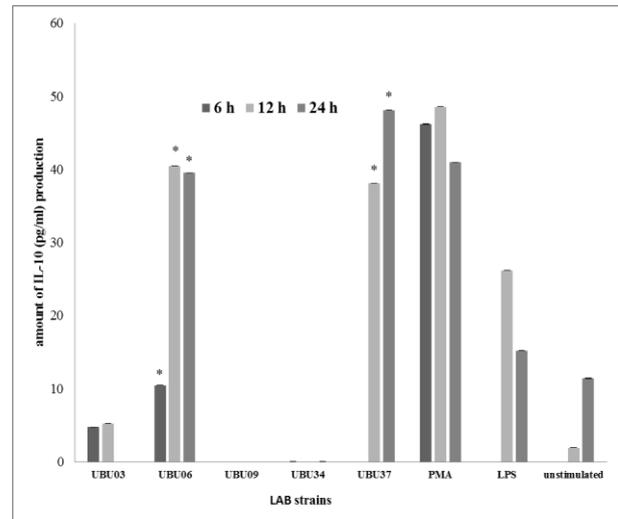


Figure 4: IL-10 production at 6 h, 12 h and 24 h from THP-1 cells after stimulation with live LAB, PMA, LPS and from unstimulated cells. Results are presented as mean \pm S.D.

*p-value < 0.05 indicates statistically significant difference when comparing stimulated with unstimulated condition.

Table 2: Summary of the ability of five *Lactobacillus rhamnosus* strains to induce IL-6, IL-10 and TNF- α production from the THP-1 cell line.

LAB strains	IL-6	IL-10	TNF- α
UBU03	+	-**	-
UBU06	+	+	-
UBU09	+	-	-
UBU34	+	-	+
UBU37	+	+	+

* + means significantly elevated levels of cytokine production at one or more time points

** - means non-significant levels or undetected cytokine production at all time points (6, 12, and 24 h)

DISCUSSION

Adhesion by probiotic bacteria to host cells is the first crucial step for their colonization of the intestinal tract, where they can exert their antimicrobial activities. These activities include exclusion of pathogens by competing for or blocking their binding sites in the mucosa (Wang *et al.*, 2014) and/or certain immunomodulatory effects (Alander *et al.*, 1999; Rocha *et al.*, 2012). Investigation of the adhesive capacity of bacteria is difficult *in vivo*. However, measurement of their ability to adhere to the human intestinal epithelium is important when evaluating probiotic strains (Tuomola and Salminen, 1998). Hence, the *in vitro* model using the Caco-2 cell line has become widely used (Dimitrov *et al.*, 2014). In this study, all five *L. rhamnosus* strains exhibited high capacity to adhere to Caco-2 cells. Indeed, strains UBU09, UBU34 and UBU37 showed higher percentages of adhesion than did the reference

strain, *L. rhamnosus* GG. Although other studies have obtained very similar results with respect to Caco-2 cells (Jacobsen *et al.*, 1999; Tuomola *et al.*, 1999; Rohani *et al.*, 2015), Jensen *et al.* found that their strain of *L. rhamnosus* showed poor adhesive capacity to Caco-2 and HT-29 cells but higher adhesion to the mucin-producing cell line LS174T (Jensen *et al.*, 2012). Thus, cell-adhesion capacity appears to depend on species and strain of bacterium, and on the cell line tested.

Several *Lactobacillus* species isolated from human and animal feces exert antimicrobial activity against food borne pathogens such as *Salmonella typhimurium*, enterotoxigenic *E. coli* and *Enterococcus faecalis* by competition for attachment sites in the intestinal tract (Todoriki *et al.*, 2001). We evaluated all five strains for their competitive adhesion ability against enteropathogenic *E. coli* O157:H7. All except one (UBU03) showed the ability to inhibit the adhesion of *E. coli* O157:H7 to Caco-2 cells. Although UBU03 cannot competitively inhibit the adhesion of *E. coli* O157:H7 to Caco-2 cells, we showed previously that UBU03 can exert antimicrobial activity against both *E. coli* O157:H7 and *V. cholerae* (Panya *et al.*, 2016). Besides competitive inhibition, Hirano *et al.* (2003) demonstrated that *L. rhamnosus* (ATCC 53103), although unable to inhibit the adhesion of enterohaemorrhagic *E. coli* (EHEC) to human colon epithelial cell line C2BBE1 (a polarized subline of Caco-2 cells), it could inhibit the internalization of EHEC to C2BBE1 cells and led them to suggest that the interaction between *L. rhamnosus* and the host cell might be responsible for this (Hirano *et al.*, 2003).

All five *L. rhamnosus* strains that we tested can stimulate production of certain proinflammatory cytokines (IL-6 and/or TNF- α) by THP-1 cells. UBU34 stimulated the highest level of TNF- α approximately 200-fold greater than that produced by unstimulated THP-1 cells. However, none of the five bacterial strains could stimulate production of IL-12. All five strains, but especially UBU34, may have potential for application in situations favoring inflammatory reaction, but not for Th1 response. On the contrary, LGG can preferentially stimulate the production of both IL-12 and TNF- α from dendritic cells, macrophages and monocytes (all associated with Th1 response) from healthy subjects (Fong *et al.*, 2015). The authors suggested that LGG might have application in improving and preventing the development of Th2-mediated inflammatory diseases and against infections.

IL-6 is a pleiotropic cytokine possessing both pro- and anti-inflammatory properties but it is mostly regarded as a pro-inflammatory cytokine (Scheller *et al.*, 2011). In addition, IL-6 also plays an important role in mucosal immunity as gut macrophages help gut B cells to differentiate to plasma cells which generate secretory IgA releasing in the intestinal lumen (Chang *et al.*, 2015). Interestingly, our UBU03 did not exhibit competitive adherence inhibition against *E. coli* O157:H7, yet is known to exhibit antimicrobial activities against both *E. coli* O157:H7 and *V. cholerae* (Panya *et al.*, 2016) and can stimulate THP-1 cells to produce very high levels of IL-6 (more than 200-fold) relative to unstimulated controls.

Thus, UBU03 may have application for facilitating mucosal immunity against infection.

IL-10 is a regulatory cytokine that has pleiotropic effects in immunomodulation and inflammation. As an anti-inflammatory cytokine, it can inhibit the synthesis of proinflammatory cytokines. On the other hand, it can also stimulate B-cell proliferation and differentiation and favour humoral immunity (Volk *et al.*, 2001). LABs with the ability to induce this anti-inflammatory cytokine have applications in allergic diseases such as asthma, eczema and allergic rhinitis (Wu *et al.*, 2016). LGG can stimulate a low level of IL-10 from THP-1 cells (Jensen *et al.*, 2015) where as other *L. rhamnosus* strains isolated from humans can induce a high level of IL-10 and IL-12 from human peripheral blood mononuclear cells and monocytes (Hessle *et al.*, 1999). In our study, UBU06 and UBU37 could stimulate a considerable amount of IL-10 from THP-1. Furthermore, both strains could also stimulate the production of IL-6, and UBU37 could additionally stimulate production of TNF- α . Where any LAB can stimulate the production of two or more cytokines with antagonistic actions, further study, especially *in vivo*, is required to evaluate the net immunomodulatory outcome. Although the precise mechanisms involving in *in vitro* cytokine induction profiles of different probiotic strains are still unclear, it has been demonstrated that different bacterial cell components may account for such differences (Ashraf *et al.*, 2014; Jeong *et al.*, 2015).

In conclusion, all five *L. rhamnosus* strains from breast-fed infants exhibited high adhesive ability to the Caco-2 cell line and all tested strains except UBU03 could competitively inhibit the adhesion of EPEC to Caco-2 cells. In assays of their immunomodulatory activity using the THP-1 cell line, it was found that the most potent inducers of IL-6, IL-10 and TNF- α production were UBU03, UBU37 and UBU34, respectively. None of the tested strains could induce IL-12 production. Induction of multiple cytokines occurred in some cases: UBU06 induced production of IL-6 and IL-10, UBU34 induced IL-6 and TNF- α and UBU37 induced IL-6, IL-10 and TNF- α . Our findings on the adhesive and immunomodulatory properties of five strains of probiotic *L. rhamnosus* set the scene for further studies on animal models before translation to proper clinical applications.

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CONFLICT OF INTEREST

The authors have no conflict of interest to disclose.

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