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Original Article

In vitro adhesion property and competition against enteropathogens of *Lactobacillus* strains isolated from Thai infants

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Abstract

Adhesion to the intestinal epithelium is considered to be one of the selection criteria for probiotics strain. In this study, the adhesion of four different *Lactobacillus* strains with potential probiotics properties, *i.e. L. paracasei* MSMC39-1, *L. casei* MSMC39-3, *L. salivarius* MSMC105-3 and *L. plantarum* MSMC171-1, was studied using Caco-2 cell line as an *in vitro* model for intestinal epithelium. Among four different *Lactobacillus* strains, *L. salivarius* MSMC105-3 was the most adhesive strain showing about 3.5 percent of adhesion index. Thus, this strain was selected to examine for its ability to inhibit the adhesion of pathogenic *Salmonella* Typhi DMST5784 and *Shigella dysenteriae* DMST15111 to Caco-2 cells. The results showed that *L. salivarius* MSMC105-3 whole cell and its cell-free culture supernatant could inhibit the adhesion of pathogens. The results from this study indicated that both *L. salivarius* MSMC105-3 itself and its substances secreted into culture supernatant had the ability to reduce the adhesion of enteropathogens to Caco-2 cells.

Keywords: probiotics, *Lactobacillus*, Caco-2 cell, adhesion, competition, *Salmonella* Typhi, *Shigella dysenteriae*

1. Introduction

Probiotics are viable microbes which confer a health benefit to the host when administered in adequate amount (Food and Agricultural Organization, World Health Organization, 2002). Bacteria in the genera *Bifidobacterium* and *Lactobacillus* are widely used as probiotics and included in various food products (Bermudez-Brito *et al*., 2012). Several criteria have been proposed for the selection of potential probiotics (Collin *et al*., 1998). One of the main criteria for the selection of a potential probiotic is its ability to adhere to the intestinal mucosa. It is believed that this property helps increase the persistence of probiotics in the host intestine and allows the probiotics to exert their effects (Kolida *et al*., 2006). However, due to the difficulty to investigate the adhesion of probiotics to host epithelial cells *in vivo*, the preliminary studies of adherent strain are extensively based on *in vitro* adhesion models which involve tissue cultures of human colon carcinoma cell lines, such as Caco-2 and HT-29 (Gopal *et al*., 2001). These cell lines spontaneously differentiate under standard culture condition and the differentiated cells express characteristics of mature enterocytes, including polarization, a functional brush border and apical intestinal hydrolases (Pinto *et al*., 1983).

The adhesion to epithelial cells is also a critical step for pathogenic bacteria because it allows the release of enzymes and toxins into the target cell to facilitate the invasion (Jankowska *et al*., 2008). There are many mechanisms that the host utilizes to prevent gastrointestinal epithelial cells from an invasion of pathogenic bacteria. One of these mechanisms is the competition of microbiota for adhesion sites with pathogens to reduce infections

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(Baccigalupi *et al*., 2005). It has been shown in several studies that probiotics can prevent the attachment of pathogens to epithelial cells and protect them from infections (Gomes *et al*., 2012; Hudault *et al*., 1997).

The aim of this study was to investigate the adhesion property of four *Lactobacillus* strains isolated from newborn feces to Caco-2 cells monolayer and adhesion competition against pathogenic bacteria. These four strains of lactobacilli were selected to use in this study because they were previously shown some promising probiotic characterristics *in vitro*, *i.e.* acid and bile tolerance, ability to modulate cytokine production, and ability to inhibit the growth of cancer. Thus, these strains are excellent candidates to be further investigated for other probiotic properties and can be developed to be good probiotics in the future.

2. Materials and Methods

2.1 Bacterial strains and growth conditions

Four *Lactobacillus* strains, isolated from neonatal human feces (ethical approval number SWUEC37/2551), were used in this study, *i.e. L. paracasei* MSMC39-1, *L. casei* MSMC39-3, *L. salivarius* MSMC105-3 and *L. plantarum* MSMC171-1. Two strains, MSMC39-1 and MSMC39-3, were previously shown to have the ability to modulate the TNF-α production (Ladda *et al*., 2015). Another two strains, MSMC105-3 and MSMC171-1, were shown to have the potential to inhibit the growth of cancer cells (publication is in process). *L. casei* Shirota (Yakult®, Yakult) was used as a reference strain (Botes *et al*., 2008; Tuomola and Salminen, 1998). All *Lactobacillus* strains were grown in MRS broth (de Man-Rogosa-Sharpe) or on MRS agar (HiMedia Laboratories, India) under anaerobic conditions at 37°C. For competition assay, two strains of enteropathogenic bacteria, *i.e. Salmonella* Typhi DMST5784 and *Shigella dysenteriae* DMST15111 were used. The pathogenic bacteria were cultured in Brain-heart infusion broth (BHI) or on BHI agar (HiMedia Laboratories, India) at 37°C.

2.2 Caco-2 cell culture

The human intestinal epithelial cell line, colorectal adenocarcinoma (Caco-2) (ATCC HTB-37) was purchased from the American Type Culture Collection (USA). The cell were cultured in Dulbecco's modified Eagle's minimal essential medium (DMEM; Gibco-Invitrogen, USA) supplemented with 10% (v/v) heat-inactivated (56°C, 30 min) fetal bovine serum (FBS; Gibco-Invitrogen, USA) and 1% Pennicillin-Streptomycin (10,000U/ml) (Gibco-Invitrogen, USA). The cell cultures were incubated at 37°C in a humidified atmosphere containing 5% CO_2. The cell culture medium was changed every 2 days, and the cells were subcultured at 80% confluent. For adhesion and competition-based adhesion assays, Caco-2 cells were seeded ($1x10^5$ cells/ml) in 24-well plates and incubated to obtain confluence at 37°C in a humidified atmosphere containing 5% CO_2, prior to the assays. The cell culture medium was changed every day.

2.3 Preparation of cell-free culture supernatant

Cell-free culture supernatant (CS) was prepared as previously described (Taweechotipatr *et al*., 2009). Briefly, *L. salivarius* MSMC105-3 was grown overnight in MRS broth under anaerobic conditions at 37°C. Overnight culture was diluted to the concentration of $1x10^8$ cfu/ml in MRS broth and further grown anaerobically for 48 h. Cell-free culture supernatant was collected by centrifugation at 4000 x *g* for 10 min at 4°C, filter-sterilized using a 0.22 µm pore size filter (Millipore, USA), and concentrated by speed-vacuum drying (Rotational Vacuum Concentrator RVC2-18, Germany). The pellet was resuspended in an equal volume of serum-free DMEM and stored at -20°C until used.

2.4 Adhesion assay

The lactobacilli cultures in MRS broth were harvested by centrifugation at 4000 x g for 10 min at 4°C, washed twice with PBS and diluted to the concentration of $1x10^8$ cfu/ml in serum-free DMEM (without antibiotics). Meanwhile, Caco-2 cells were also washed twice with PBS to remove antibiotics before the bacterial suspension was added. One milliliter of inoculum with defined bacteria concentration was added to each well of 24-well plate, and the plates were incubated for 1 h at 37°C in a humidified atmosphere containing 5% CO_2. After that, the supernatants were removed and wells were gently washed four times with PBS to remove non-attached bacteria. Following the last wash, Caco-2 cells were detached with 0.25% trypsis-EDTA solution (Gibco-Invitrogen, USA). Cells were lysed with sterile distilled water for 5 min. The adherent bacteria were counted by plating the serial 10-fold dilution of the suspensions using agar plates. Adhesion data were expressed as the percentage of bacteria adhered compared to the total of bacteria added (cfu bacteria adhered/cfu bacteria added). The *Lactobacillus* strain that showed the greatest adhesion to Caco-2 was selected for further study in competition-based adhesion assays.

2.5 Competition-based adhesion assays

The enteropathogenic bacteria, *Salmonella* Typhi DMST5784 and *S. dysenteriae* DMST15111, and *L. salivarius* MSMC105-3 were cultured in BHI broth or MRS broth. Bacteria were harvested by centrifugation at 4000 x g for 10 min at 4°C, washed twice with PBS and diluted to the concentration of $1x10^8$ cfu/ml in serum-free DMEM (without antibiotics). Different types of experiments were performed: (i) competition assay: 0.5 ml of either *Salmonella* Typhi or *S. dysenteriae* was mixed together with 0.5 ml of *Lactobacillus*, and added onto Caco-2 cell monolayer and coincubated for 1 h; (ii) exclusion assay: Caco-2 cells were first pre-incubated with 0.5 ml of *Lactobacillus* for 1 h and then 0.5 ml of *Salmonella* Typhi or *S. dysenteriae* was added onto Caco-2 cells and incubated for 1 h; (iii) displacement assay: 0.5 ml of *Salmonella* Typhi or *S. dysenteriae* was added onto Caco-2 cells and incubated for 1 h before challenged with *Lactobacillus* for 1 h. To evaluate the effect of cell-free supernatant on the competition with the enteropathogenic

bacteria, cell-free supernatant of *L. salivarius* MSMC105-3 was added to Caco-2 cell instead of bacterial cells. The plates were incubated for 2 h at 37°C in a humidified atmosphere containing 5% CO_2. After that, the supernatants were removed and wells were gently washed four times with PBS to remove non-attached bacteria. Following the last wash, Caco-2 cells were detached with 0.25% trypsin-EDTA solution and cells were lysed with sterile distilled water for 5 min. The adherent bacteria were counted by plating the serial 10-fold dilution of the suspensions using agar plates. Adhesion data were expressed as the percentage of bacteria adhered compared to the total of bacteria added (cfu bacteria adhered/cfu bacteria added)

2.6 Statistical analysis

Data are shown as mean±SE. Data were analyzed by one-way analysis of variance (ANOVA) followed by Turkey posthoc test or Mann-Whitney U test (GraphPad Prism version 5.01, GraphPad Software, USA). A p-value ≤ 0.05 was considered statistically significant.

3. Results

3.1 Adhesion of *Lactobacillus* to Caco-2 cells

Adhesion to intestinal epithelial cells is considered to be the key process for probiotics to survive and colonize the gastrointestinal tract and also related to the exclusion of intestinal pathogens. In this study, adhesion of four selected strains of *Lactobacillus* was evaluated using the *in vitro* Caco-2 cell line model (Figure 1).

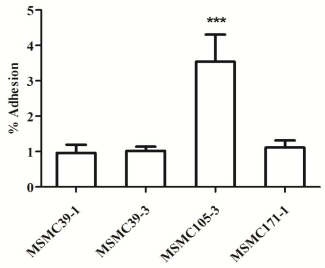

Figure 1. Adhesion level of four *Lactobacillus* strains whole cells to the Caco-2 cells. Experiments were performed three times, in duplicate. The values represent the mean and SE; ***P < 0.001.

The results presented in Figure1 show that all strains of *Lactobacillus* demonstrated an adherence to Caco-2 monolayer. The *L. salivarius* MSMC105-3 showed the highest adhesion ability (3.54±0.77%). The adhesion level of this strain was statistically significant different from other *Lactobacillus* strains (P < 0.05). The adhesion level of *L. plantarum* MSMC171-1, *L. casei* MSMC39-3 and *L. paracasei* MSMC39-1 were 1.12±0.2%, 1.02±0.12% and 0.96±0.23%, respectively. The statistical analysis did not show any significant differences between their adhesive abilities. In addition, the reference strain (*L. casei* Shirota) was shown to have constant level of adhesion in all experiments (1.54±0.46%). This adhesion level of reference strain was in consistent with the results from previously studies which *Kankaanpaa et al.* (2001) report adhesion yield of *L. casei* Shirota at about 1.6% and Lewandowska *et al.* (2005) reported at about 2%.

3.2 Effect of *L. salivarius* MSMC105-3 on inhibition of enteropathogenic bacteria adhesion to Caco-2 cells

As *L. salivarius* MSMC105-3 showed the highest adhesion level to Caco-2, this strain was selected to study for its effect on the adhesion of enteropathogenic bacteria, *Salmonella* Typhi and *S. dysenteriae*, to Caco-2 cells. Competition, exclusion and displacement assays were performed to investigate the abilities of *L. salivarius* MSMC105-3 in reducing the adherence of enteropathogens to Caco-2 cells (Figure 2). In exclusion assay, the adherence of both *Salmonella* Typhi and *S. dysenteriae* was decreased significantly by *L. salivarius* MSMC105-3 ($P < 0.001$). Specifically, the *Lactobacillus* reduced about 10-fold adhesion of *Salmonella* Typhi and about 5-fold adhesion of *S. dysenteriae* to Caco-2 cells. Similar to the results from exclusion assay, the *L. salivarius* MSMC105-3 could reduce the adhesion level of enteropathogens but to the lesser extent than in the exclusion assay (about 3-fold and about 1.5-fold for *Salmonella* Typhi and *S. dysenteriae*, respectively). In contrast, *L. salivarius* MSMC105-3 showed no activity in reducing the adhesion of enteropathogens in displacement assay.

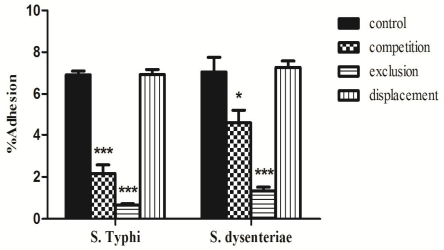

Figure 2. Effect of *L. salivarius* MSMC105-3 treatment on *S.* Typhi and *S. dysenteriae* adhesion to Caco-2 cells. *L. salivarius* MSMC105-3 was used in competition, exclusion and displacement assays. Experiments were performed three times, in duplicate. The graph showed adhesion level of the enteropathogens to Caco-2 cells. The values represent the mean and SE; *P < 0.05; ***P < 0.001.

3.3 Effect of cell-free supernatant from *L. salivarius* MSMC105-3 to adhesion of enteropathogenic bacteria

To study whether the substances secreted by *L. salivarius* MSMC105-3 have an effect on the adhesion of enteropathogens, the cell-free supernatant from *L. salivarius*

MSMC105-3 was co-incubated with enteropathogens and Caco-2 cells (Figure 3). The co-incubation of cell-free supernatant with enteropathogens led to a significant decrease of adhesion of enteropathogens to Caco-2 cells ($P < 0.01$). These results indicated that not only *L. salivarius* MSMC105-3 itself but also the substances secreted by *L. salivarius* MSMC105-3 to the medium might counteract the adhesion of enteropathogens to Caco-2 cells.

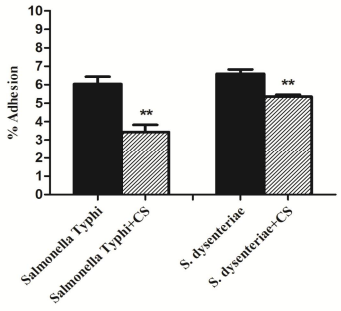

Figure 3. Effect of cell-free culture supernatant of *L. salivarius* MSMC105-3 treatment on *S.* Typhi and *S. dysenteriae* adhesion to Caco-2 cells. Experiments were performed three times, in duplicate. The graph showed adhesion level of the enteropathogens to Caco-2 cells. The values represent the mean and SE; **P < 0.01.

4. Discussion

The ability of probiotics to adhere to the epithelial cells of host intestinal tract is crucial for establishing colonization and exerting health benefit to the host. Therefore, adhesion to epithelial cells has been considered as one of the selection criteria for probiotics strains. In the present study, the Caco-2 cell line was used as a model for the investigation of lactobacilli adhesion to the intestinal epithelium because it can differentiate and closely resemble enterocytes of the human small intestine (Ouwehand *et al*., 1999). The adhesion of four *Lactobacillus* strains isolated from newborn feces to Caco-2 cells was investigated. Among four selected strains of *Lactobacillus*, *L. salivarius* MSMC105-3 was the most adhesive strain. This strain showed adhesion level approximately 3.5 percent while the rest showed only about 1 percent of adhesion. The adhesion level observed for this strain is higher than the adhesion level of *L. salivarius* C3 (Kirtzalidou *et al*., 2011), *L. salivarius* SMXD51 (Messaoudi *et al*., 2012) and *L. salivarius* LS-33 (Collado *et al*., 2007). The adhesion of *Lactobacillus* to epithelial cells may occur through the specific or non-specific adhesion between epithelial cells and bacterial surface components (Kleerebezem *et al*., 2010). Several surface molecules have been reported to contribute to specific and/or non-specific adhesion of lactobacilli to host epithelial cells, for example, mucus-binding proteins, mannose-specific adhesions, S-layer proteins, lipoteichoic acid and exopolysaccharides (Kleerebezem *et al*., 2010; Lebeer *et al*., 2008). Dunne *et al*. (2004) has shown that the pretreatment of *L. salivarius* UCC118 cells with proteolytic enzymes abolished adhesion, indicating the involvement of surface protein(s) such as bacterial adhesion(s) in the attachment of this strain onto the epithelial cells. However, the adhesion mechanism(s) of *L. salivarius* MSMC105-3 was not investigated in this study. Further study is needed to search for the mechanism(s) this strain using to adhere to epithelial cells. Adhesion to host epithelial cell is also a key process for pathogenic bacteria since it allows bacteria to release enzymes and toxins to initiate the invasion into host cells and cause diseases. Competition of probiotics with the pathogens for adhesion and colonization could prevent the host from an infection. Several reports have documented the ability of probiotics to inhibit the adhesion and invasion of pathogenic bacteria (Gueimonde *et al*., 2006; Ingrassia *et al*., 2005; Moroni *et al*., 2006; Ren *et al*., 2013; Resta-Lenert & Barrett, 2003). In this study, *L. salivarius* MSMC105-3 showed the highest adhesion level among the four selected strains. This result showed the potential role in the competitive exclusion of pathogens. Therefore, the effect of this strain against enteropathogenic bacteria was tested. The result demonstrated that *L. salivarius* MSMC105-3 could reduce the adhesion level of *Salmonella* Typhi and *S. dysenteriae* to the highest degree in exclusion assays and to the lesser extent in competition assay, but it could not inhibit the adhesion of enteropathogens in displacement assay. Similar results were found in *L. salivarius* CRL1328 which can inhibit the adherence of *Staphylococcus ayreus* and *Streptococcus agalactiae* to vaginal epithelial cells in competition, exclusion, and displacement assay. However, the inhibition index was highest in exclusion assay and weakest in displacement assay (Zarate & Nader-Macias, 2006). Ren *et al*. (2013) also showed the ability of *L. salivarius* CICC23174 to inhibit adhesion of *Bacillus cereus*, *Escherichia coli* and *Staphylococcus aureus* to Caco-2 cell in the competition assay. In addition, the cell-free culture supernatant from probiotics has also been reported to have an inhibition effect on the adhesion or invasion of enteropathogenic bacteria. Pre-treatment of *E. coli* O157:H7 with the cell-free culture supernatants from *L. acidophilus* HN017, *L. rhamnosus* DR20 or *B. lactis* DR10 could reduce of the adhesion and invasion of *E. coli* O157:H7 (Gopal *et al*., 2001). The cell-free culture supernatant from *L. paracasei* IBB2588 was also shown to inhibit the adhesion of *S. enterica* KOS1663 to epithelial cells (Jankowska *et al*., 2008). The protein secreted by *L. salivarius* Lv72 can abrogate the adhesion of *Actinomyces neuii* to epithelial cells (Martin *et al*., 2012). The resembling effect was also found in this study. The cell-free supernatant from *Lactobacillus* MSMC105-3 could reduce pathogen adhesion level to Caco-2 cells; nevertheless, the effect was weaker than that of whole cell *Lactobacillus*. Taken together, these finding imply that the ability of *L. salivarius* MSMC105-3 to inhibit adhesion of enteropathogens may involve not only competition for epithelial cell receptors but also some substances secreted by *Lactobacillus* (not defied in this study) may act as inhibitors of pathogens adhesion.

Even though *L. salivarius* MSMC105-3 did not show a very high adhesion index, it still showed the capability to inhibit the adhesion of enteropathogenic bacteria that might be used for prevention of gastrointestinal tract infection. Furthermore, this strain has been shown in our laboratory to exhibit other promising probiotic properties as it could inhibit the growth of cancer cells, and well tolerate exposure to acid and bile (Data is in the process of publication). Therefore, this strain is considered to be a good candidate for further search for other probiotic characteristics and for developing to be the good probiotics in the future.

Although *in vitro* experiments are essential to provide the understanding of mechanism of adhesion and competition of probiotics, it is important to note that *in vitro* results of bacteria adhesion are difficult to represent what happen *in vivo* studies. This is because different factors presenting in dynamic environment of the gastrointestinal tract (e.g. cell-communication molecules, resident microbiota and peristaltic flow) can modify the bacteria adhesion (Lebeer *et al*., 2008). Thus, this observation *in vitro* has to be confirmed *in vivo.*

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