

## Adhesion Properties of Indigenous Dadih Lactic Acid Bacteria on Human Intestinal Mucosal Surface

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**ABSTRACT :** Dadih is Indonesian traditional fermented buffalo milk believed by the natives to have beneficial effects on human health. This may be due to the probiotic properties possessed by the lactic acid bacteria (LAB) involved in its fermentation process. It was discovered that ten strains of dadih lactic isolates possessed some probiotic properties *in vitro*. In this study, the adhesion properties of dadih LAB, in comparison with documented probiotic strains, were investigated *in vitro* by using mucin extracted from human faeces and Caco-2 cells as the models for human intestinal mucosal surface and intestinal cells respectively. The adhesion results showed the distinction of *Lactobacillus reuteri* IS-27560 in adhering to both mucus layer and Caco-2 cells. The competition assay for adhesion to the mucus layer between dadih LAB and selected pathogens indicated the competence of *Lactococcus lactis* IS-16183 and *Lactobacillus rhamnosus* IS-7257 in significantly inhibiting the adhesion of *Escherichia coli* O157:H7. Accordingly, these two strains may be potential candidates for use as probiotic strains. Overall, the adhesion properties of all dadih LAB strains were relatively comparable to that of *Lactobacillus casei* Shirota and *Lactobacillus rhamnosus* GG, the documented probiotic strains. (*Asian-Aust. J. Anim. Sci.* 2006. Vol 19, No. 5 : 751-755)

**Key Words :** Dadih, Lactic Acid Bacteria, Adhesion, Probiotics, Mucin, Caco-2 Cells

### INTRODUCTION

The emerging of probiotics these days is predominantly driven by the demands of consumers for foods that are able to provide other benefits for health beyond their basic nutritional value. Salminen et al. (1999) defined probiotics as microbial cell preparations or components of microbial cells that have a beneficial effect on the health and well-being of the host. Many lactic acid bacteria (LAB) strains have been classified as probiotics due to their beneficial effects towards human health; in particular, the gastrointestinal (GI) tract. Those reported include prevention of colon cancer, reduction of cholesterol levels, inhibition of pathogenic microflora, stimulation of immune response, reduction of constipation, enhancement of lactose digestion in lactase deficient subjects as well as alleviation of food allergy (Salminen et al., 1998; Kaur et al., 2002; Ouwehand et al., 2002; Saarela et al., 2002).

There are a few criteria in selecting potential probiotic strains as illustrated by Saarela et al. (2000), and one of them is the ability of the bacteria to adhere to epithelial cell surfaces. Adhesion of probiotic strains on mucosal surface is one of the most important probiotic properties because it is often recognized as the prerequisite for colonization of the human GI tract (Beachey, 1981). Besides, adhesion is

also a means of competitive exclusion of the pathogenic bacteria from the intestinal epithelium; and the adherent bacteria strains induce immune effects more effectively and stabilize the intestinal mucosal barrier from invasion by pathogens (Salminen et al., 1996).

Dadih is Indonesian traditional fermented buffalo milk and is believed by the natives to be beneficial for consumers' health. This might be contributed by the probiotic properties exerted by the indigenous lactic acid bacteria present in dadih. Some studies on probiotic properties of indigenous lactic acid bacteria isolated from dadih have shown to exhibit antimutagenic, hypocholesterolemic, acid and bile tolerance as well as antipathogenic properties (Hosono et al., 1990; Surono and Hosono, 1996; Surono, 2003; Pato et al., 2004).

In this study, the adhesion properties of 10 LAB strains isolated from dadih on human intestinal mucosal surface were investigated. Two probiotic strains, *Lactobacillus casei* strain Shirota (LCS) and *Lactobacillus rhamnosus* strain GG (LGG), were used for comparison study. The adhesion assays were carried out *in vitro* by using mucin extracted from human faeces and Caco-2 cells as the models for intestinal surface and intestinal cells respectively. Furthermore, the ability of dadih LAB to compete with the intestinal pathogens, namely *Escherichia coli* O157:H7, *Salmonella typhimurium* E10 and *Helicobacter pylori*, in adhesion on mucin was also observed.

### MATERIALS AND METHODS

#### Bacterial strains

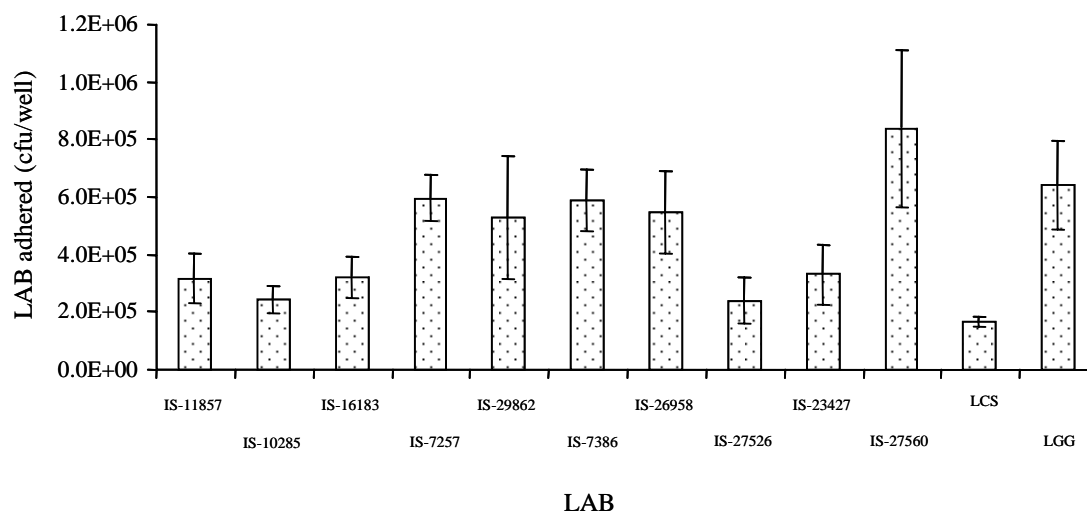
The indigenous LAB were isolated from Bukit Tinggi

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**Figure 1.** Adhesion of LAB on Caco-2 cells ( $5 \times 10^7$  cfu/well added): *Lactococcus lactis* subsp *lactis* strains IS-11857, IS-10285, IS-16183, IS-29862, IS-7386; *Lactobacillus rhamnosus* strain IS-7257; *Lactobacillus brevis* strains IS-26958, IS-23427; *Lactobacillus reuteri* IS-27560; and *Enterococcus faecium* strain IS-27526. The vertical bars represent the standard deviation of the measurements.

dadih and identified by API CH 50 (BioMerieux, France). They were *Lactococcus lactis* subsp *lactis* strains IS-11857, IS-10285, IS-16183, IS-29862 and IS-7386; *Lactobacillus rhamnosus* strain IS-7257; *Lactobacillus brevis* strains IS-26958 and IS-23427; *Lactobacillus reuteri* IS-27560; and *Enterococcus faecium* strain IS-27526. The probiotic strains *Lactobacillus casei* strain Shirota (isolated from Yakult cultured milk) and *Lactobacillus rhamnosus* strain GG (ATCC 53103), together with the pathogens *Escherichia coli* O157:H7, *Salmonella typhimurium* E10 (NCTC 8391) and *Helicobacter pylori* (ATCC 37504) were obtained from Department of Microbiology, National University of Singapore. The lactic acid bacteria were cultured in de Man, Rogosa and Sharpe (MRS) broth (Merck, Germany) while Nutrient broth (Oxoid, England) was used to culture *E. coli* O157:H7 and *S. typhimurium* E10. The incubation was carried out at 37°C in air atmosphere for 18±2 h before the adhesion study. *H. pylori* was cultured in the Brain Heart Infusion (BHI) broth (BBL, USA) supplemented with 0.4% (wt/vol) yeast extract (Oxoid, England) and 10% (vol/vol) horse serum. It was incubated at 37°C in the microaerophilic condition for 3 days prior to adhesion study.

#### Caco-2 cells

The Caco-2 cells (obtained from American Type Culture Collection, Rockville, USA) were cultured in the Minimal Essential Medium (MEM) with Earle's salts that contained 25 mM glucose, supplemented with 20% (vol/vol) heat-inactivated (30 min, 56°C) Fetal Bovine Serum, 2 mM L-glutamine, Penicillin G (100 U/mL), Streptomycin (100 µg/mL), 1 mM sodium pyruvate, 0.1 mM non-essential amino acids (all from GIBCO-BRL, USA) and 0.15% NaHCO<sub>3</sub> (Merck, Germany). The cells were cultured in the

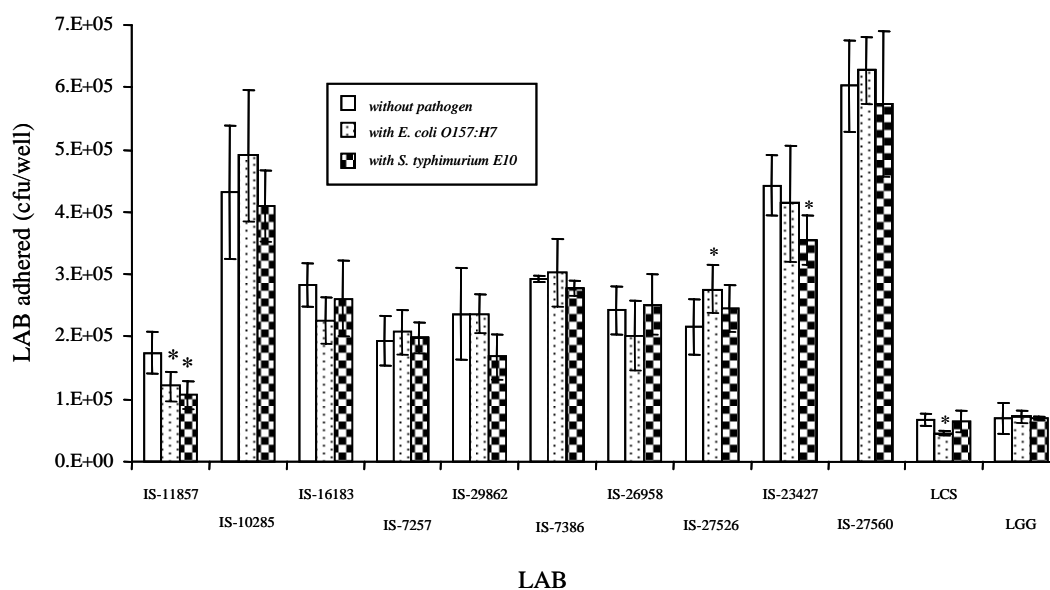
75 cm<sup>2</sup> tissue culture flask (Falcon, England) for 14±2 days at 37°C in 5% (vol/vol) CO<sub>2</sub> in air atmosphere to become a confluent layer. For adhesion assays, monolayer Caco-2 cells were transferred to 24-well tissue culture plates (Nunc, Denmark) at a concentration of approximately  $1 \times 10^5$  viable cells per well in 1 mL of culture medium. The cells were cultured for another 14±2 days to obtain their confluence prior to adhesion assay. The culture medium was replaced every 2 to 3 days.

#### Human intestinal mucin

The mucin was extracted from faeces of healthy adults by extraction and dual ethanol precipitation (Kirjavainen et al., 1998). Prior to adhesion assays, the crude mucin extract was dissolved in Hank's balanced salt solution (GIBCO) containing HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid, Sigma) at pH 7.4-7.6 (HH buffer) to remove intestinal contents. A hundred µl of mucin dissolved in HH buffer (0.05% wt/vol) was then passively immobilized to each well of polystyrene Maxisorp microtiter plate wells (Nunc, Denmark) for 18±2 h at 4°C in air atmosphere.

#### In vitro adhesion assays

The adhesion assays on mucus layer and Caco-2 cells were carried out by incubating the radioactive labelled bacterial cells (labelled with 113 Ci mmol<sup>-1</sup> of [methyl, 1', 2'-<sup>3</sup>H] Thymidine obtained from Amersham, UK) on immobilized mucin and on Caco-2 cells respectively for 1 h at 37°C. After being washed to remove the free bacterial cells, the adhesions of LAB on mucus layer and Caco-2 cells were determined from their radioactivity measurement by the scintillation counter (Beckman-LS-6500). Similarly,



**Figure 2.** Adhesion of LAB on mucin ( $5 \times 10^6$  cfu/well added): *Lactococcus lactis* subsp *lactis* strains IS-11857, IS-10285, IS-16183, IS-29862, IS-7386; *Lactobacillus rhamnosus* strain IS-7257; *Lactobacillus brevis* strains IS-26958, IS-23427; *Lactobacillus reuteri* IS-27560; and *Enterococcus faecium* strain IS-27526. The vertical bars represent the standard deviation of the measurements. (\*) indicates that it is significantly different from adhesion without pathogen at 95% confident level ( $p < 0.05$ ).

the competition assay in adhesion on mucus layer was performed by labelling either LAB or pathogens with radioactive  $^3\text{H}$  and incubating them on immobilized mucin for 1 h at  $37^\circ\text{C}$ . The degree of adhesion was expressed as colony forming unit per well (cfu/well). The degree of competition between LAB and pathogens was expressed in percentage of change, by determining the changes in adhesion.

### Statistical analysis

The data were analyzed by using SPSS program 11.0 for Windows. Student's t-test or the least significant difference (LSD) test after analysis of variance (ANOVA) was used to identify the differences in the competition assay. A  $p$  value  $< 0.05$  is considered statistically different at 95% confident level.

## RESULTS AND DISCUSSION

### Adhesion of LAB on Caco-2 cells

The chart in Figure 1 indicates that all dadih LAB strain had the ability to adhere on Caco-2 cells to various extents, when  $5 \times 10^7$  cfu of LAB were added to a well containing approximately  $1 \times 10^5$  viable Caco-2 cells. The number of bacteria adhered on Caco-2 cells varied for each LAB strain and it ranged from  $2.4 \times 10^5$  to  $8.4 \times 10^5$  cfu/well or around 0.5% to 1.7% of the LAB added. Among dadih LAB strains, *Lb. reuteri* IS-27560 had the highest number of bacteria adhered on Caco-2 cells. The results also showed that LGG had approximately 1% adhered bacteria on Caco-2 cells

while LCS had 0.33%. In general, dadih LABs were comparable with LGG and LCS in adhering on Caco-2 cells.

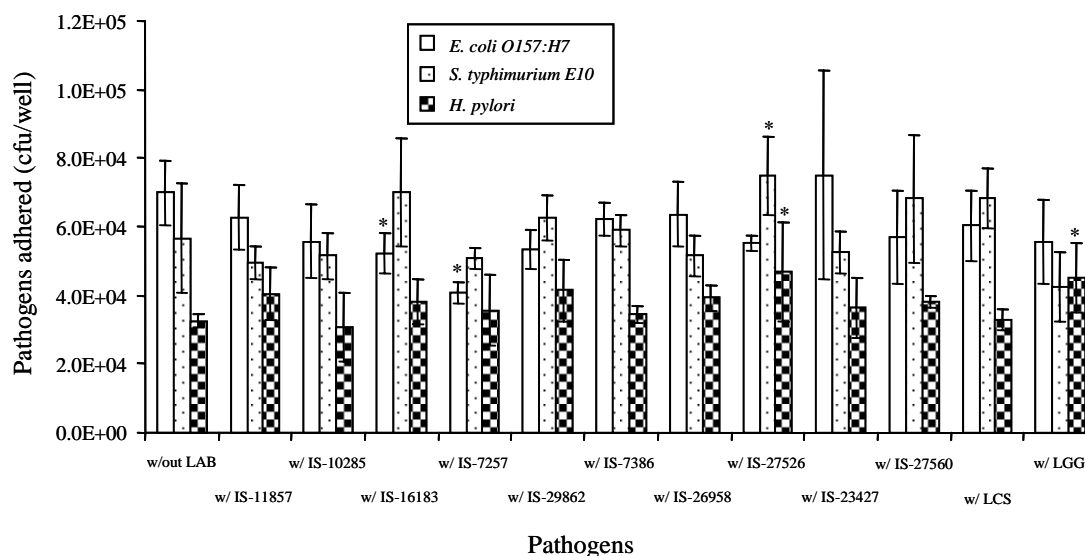
### Adhesion of LAB on mucin

Each dadih LAB strain also showed various degree of adhesion on mucin when  $5 \times 10^6$  cfu of LAB was added to a well containing immobilized mucin without the presence of pathogens. Figure 2 shows that the number of dadih LAB adhered on mucin ranged from  $1.7 \times 10^5$  to  $6.0 \times 10^5$  cfu/well or about 3.5% to 12% of the bacteria added, with *Lb. reuteri* IS-27560 as the most adherent strain. It is also shown that the adhesion on mucin of all dadih LAB strains, except *Lc. lactis* IS-11857, were significantly higher compared with that of LCS and LGG, which had about 1% adhesion.

### Competition in adhesion between LAB and pathogens on mucin

In the competition assay, the adhesions of most dadih LAB strains were generally not affected by the presence of pathogens substantially at 95% confident level ( $p > 0.05$ ) as shown in Figure 2. There was also no significant change observed in the adhesions of all LAB in the presence of *H. pylori* (data not shown). This might be due to the different sites of adherence between the pathogens and most LAB strains at the receptors on mucus layer, and they did not affect the adhesion of each other. Nonetheless, there were some LAB strains, whose adhesion changed notably in the presence of *E. coli* and *S. typhimurium* ( $p < 0.05$ ).

In the presence of *E. coli*, the adhesions of *Lc. lactis* IS-11857 and LCS were reduced significantly ( $p < 0.05$ ) when



**Figure 3.** Adhesion of pathogens on mucin: *E. coli* O157:H7, *S. typhimurium* E10 ( $5 \times 10^6$  cfu/well added), and *H. pylori* ( $5 \times 10^4$  cfu/well added). The LAB are *Lactococcus lactis* subsp *lactis* strains IS-11857, IS-10285, IS-16183, IS-29862, IS-7386; *Lactobacillus rhamnosus* strain IS-7257; *Lactobacillus brevis* strains IS-26958, IS-23427; *Lactobacillus reuteri* IS-27560; and *Enterococcus faecium* strain IS-27526. The vertical bars represent the standard deviation of the measurements. (\*) indicates that it is significantly different from adhesion without LAB at 95% confident level ( $p < 0.05$ ).

$5 \times 10^6$  cfu of radioactive-labelled LAB and equal number of *E. coli* were added to a well containing immobilized mucin. The number of *Lc. lactis* IS-11857 adhered on mucin was reduced from  $1.7 \times 10^5$  cfu/well to  $1.2 \times 10^5$  cfu/well in the presence of *E. coli* while it was from  $6.8 \times 10^4$  cfu/well to  $4.6 \times 10^4$  cfu/well for LCS. Both LAB had the reduction in adhesion of about 30% in the presence of *E. coli*. Moreover, the adhesions of *Lc. lactis* IS-11857 and *Lb. brevis* IS-23427 were reduced significantly by 40% and 20% correspondingly in the presence of *S. typhimurium* when equal amount of radioactive-labelled LAB and pathogen ( $5 \times 10^6$  cfu/well) was added. In this case, it could be hypothesized that the pathogens were competing for adhesion sites directly with those LAB strains whose adhesions on mucin extract were reduced significantly.

The effect of LAB on the adhesion of pathogens on mucin was confirmed by determining the capability of LAB to reduce the number of pathogens adhered on the mucosal surface. The pathogens were labelled with the radioactive  $^3\text{H}$ , and equal amount of LAB and pathogens was added to the mucin. Figure 3 shows that the adhesion of *E. coli* on mucus was reduced significantly from  $6.7 \times 10^4$  cfu/well to  $5.2 \times 10^4$  cfu/well and  $4.1 \times 10^4$  cfu/well in the presence *Lc. lactis* IS-16183 and *Lb. rhamnosus* IS-7257 respectively. The inhibition of adhesion might eventually prevent the invasion of the *E. coli* on the intestine since adhesion of pathogens is the primary step of pathogenesis (Ouweland et al., 1999). However, none of dadih LAB was capable of reducing the adhesion of both *S. typhimurium* and *H. pylori* notably.

Unexpectedly, the adhesion of *Enterococcus faecium* IS-27526 increased significantly ( $p < 0.05$ ) up by 33% from  $2.1 \times 10^5$  cfu/well to  $2.8 \times 10^5$  cfu/well in the presence of *E. coli* (Figure 2). On the other hand, this bacterial strain also enhanced the adhesions of *S. typhimurium* and *H. pylori* considerably up by 32% and 44% correspondingly (Figure 3). This might be due to the formation of aggregates between the dadih LAB and pathogens. This was presumed to be another mechanism by which probiotics prevent the attachment of pathogens directly on the intestinal surface (Bibiloni et al., 1999; Ouweland et al., 1999; Tuomola et al., 1999). Nevertheless, further research needs to be carried in order to confirm the ability of *Enterococcus faecium* IS-27526 to co-aggregate with pathogens.

The adhesion assay performed on mucus layer and Caco-2 cells depicted the distinction of *Lb. reuteri* IS-27560 in adhering on both substrata. The competence of both *Lc. lactis* IS-16183 and *Lb. rhamnosus* IS-7257 in competing with *E. coli* was also revealed in this study. Ultimately, the adhesion properties of dadih LAB strains were indeed comparable to that of the documented probiotic strains and hence, are potential novel probiotic strains.

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