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# Different strains of heat-killed Lactobacilli affected adhesion on Caco-2 and induced interleukin-6, interleukin-10 and interleukin-12 production

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Recently, dead cell has also been regarded as probiotics since they exhibited the beneficial effects to health. This study investigated the adhesion to Caco-2 cell and cytokine response of various heat-killed bacteria such as heat-killed *Lactobacillus acidophilus* TISTR 450 (HK-LA450), heat-killed *Lactobacillus casei* subsp. *rhamnosus* TISTR 047 (HK-LC047), heat-killed *Lactobacillus* sp., LCC1 (HK-LCC1) heat-killed *Lactobacillus* sp., LCC2 (HK-LCC2) and heat-killed *Escherichia coli* ATCC 25922 (HK-EC). The growth profiles, which was expressed as number of cells and specific growth rate, were examined to prepare dead cell from  $10^8$  log cfu of living cell. Percent adhesion of all heat-killed bacteria ranged between  $5.68 \pm 0.05$  and  $7.95 \pm 0.60\%$ . HK-LCC1 had the highest adhesive activity. HK-EC had the lowest adhesive activity. The ability of the immune cell associated with Caco-2 to respond to heat-killed bacterial was tested by the cytokine release. Heat-killed lactobacilli and HK-EC could induce cytokines including interleukin-6 (IL-6), IL-12 (proinflammatory cytokine) and IL-10 (anti-inflammatory cytokine). HK-LCC1 had the highest activity to induce IL-6 ( $4.63 \pm 0.70$  pg/ml) and IL-10 ( $255.59 \pm 1.22$  pg/ml). HK-EC could induce only IL-12 ( $2.45 \pm 0.11$  pg/ml). The percent adhesion did not relate to cytokine production of heat-killed lactobacilli (pearson's correlation level  $r = 0.39$ ,  $p = 0.01$ ). The HK-LCC1 showed a great activity to induce the immune cell response (IL-6 and IL-10). Thus, HK-LCC1 may effectively ameliorate symptoms or diseases caused by IL-10 deficiency.

**Key words:** Heat-killed lactobacilli, adhesion, Caco-2, cytokine, interleukin-6, interleukin-10, interleukin-12.

## INTRODUCTION

Probiotics are defined as live microbial food ingredients that have a beneficial effect on human health (Salminen et al., 1998). Though, live probiotics are generally recommended, health-promoting effects of inactive probiotic have also been observed (Ouwehand and Salminen, 1999). Members of the genera *Lactobacillus*

and *Bifidobacterium* are mainly used, but not exclusively, as probiotic microorganisms and a growing number of probiotic foods are available to the consumer. For example, *Lactobacillus plantarum* SS2 was used as a probiotic in fermented plant beverage with no adverse effects when male ICR mice was fed with high dose of *L. plantarum* SS2 (Duangjitcharoen et al., 2008, 2009).

However, the viability of a lactic acid bacteria is affected depending on the bacterial strain, culture phase, product pH, or concentration of sugar used as a sweetener. It is also very difficult to improve the viability of the lactic acid bacteria while keeping the original taste of the product. A dead cell of a lactic acid bacteria or a culture containing the dead cell of a lactic acid bacterium is, therefore, added to the dairy products. Thereby, it is possible to enhance the growth of a lactic acid bacteria, to shorten the time required for fermentation, and to improve the viability of a lactic acid bacterium during

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**Abbreviations:** TNF, Tumor necrosis factor; IL, interleukin; PBS, phosphate buffer saline; FPBs, fermented plant beverages; LPS, lipopolysaccharide; PGN, peptidoglycan; ELISA, enzyme linked immunosorbent assay; DMEM, Dulbecco's modified Eagle's minimal essential medium; TSB, tryptic soy broth; MRS, de Man, Rogosa, and Sharpe; HK-LP-L-137, heat-killed *L. plantarum* L-137.

storage over a long period, without affecting the flavor or production cost of the dairy product (Yoshioka et al., 2009).

Non-viable microbes have also been regarded as probiotics since they exhibited the beneficial effects to health. For instance, both live and dead lactobacilli can stimulate macrophage functions.

This stimulating activity has been reported to vary among strains of *Lactobacillus*. Previous study showed that heat-killed *L. plantarum* L-137 (HK-LP-L-137), a strain isolated from fermented food, is a potent stimulator of interleukin (IL)-12 and tumor necrosis factor (TNF)- $\alpha$  *in vitro* as well as *in vivo* in mice (Murosaki et al., 1998; Hirose et al., 2006). Murosaki et al. (1998) found that administration of HK-LP-L-137 suppressed IgE production against naturally fed antigen in a mouse model of food allergy. HK-LP-L-137 also obstructed tumor growth in mice transplanted with syngenic tumor cells.

These consequence of HK-LP-L-137 had been shown to exert through induction of T helper (Th)-1 related cytokines (Murosaki et al., 1998; Hirose et al., 2006).

Moreover, many studies have focused on the components of lactic acid bacteria that exhibit stimulatory activity on the innate immune system (Yoshimura et al., 1999). Among twenty strains of heat-killed lactobacilli, some strains of *L. plantarum* and *L. gasseri* had a higher stimulatory activity for IL-12 (p70) production than the other lactobacilli tested. However, this effect was strain dependent rather than species dependent (Sashihara et al., 2006). *In vivo* treatment of HK-LP-L-137 also increased the plasma level of IL-12 in mice (Murosaki et al., 1998). Nevertheless, much less information is available on the microbial factors that determine the ability to induce cytokines in a strain-dependent manner. Understanding the immunostimulatory activities in terms of the components of bacterial cells could provide further opportunities for utilizing such beneficial lactic acid bacteria as probiotics. Furthermore, attachment to mucosal surfaces may prolong the time probiotics can influence the host and the adhesion ability *in vitro* is regarded as an important selection criterion for potential probiotic strains (Elo et al., 1991; Chauviere et al., 1992). Adherence of probiotics to intestinal epithelial cells and ensuing temporary colonization of the gut is probably of crucial importance for their beneficial health effect.

The objective of this study is to investigate the effects of various heat-killed lactic acid bacteria on adhesion to Caco-2 and the immune response via observation of IL-6, IL-10 and IL-12.

## MATERIALS AND METHODS

### Preparation of dead cell microorganisms

*L. acidophilus* TISTR 450 and *L. casei* subsp. *rhamnosus* TISTR047 were obtained from Thailand Institute of Scientific and

Technological Research (TISTR). *Lactobacillus* sp. (LCC1) and *Lactobacillus* sp. (LCC2) were obtained from Health Product Research Unit, Chiang Mai university. They were cultured in de Man, Rogosa, and Sharpe (MRS; Labscan, Spain) broth at 37°C. Besides, *E. coli* ATCC 25922, the control, was cultured in Tryptic soy broth (TSB; Merck, Germany) at 37°C. The growth profile of each microorganism was investigated and expressed as the value of specific growth rate ( $\mu$ ). Optical density (OD<sub>600</sub> = 0.5) of bacterial suspension was calculated; the bacterial cell number from the growth curve (data not shown) gave approximately 10<sup>8</sup> cfu ml<sup>-1</sup>.

Bacterial cells were harvested and washed three times with phosphate buffer saline (PBS) solution, and suspended in distilled water. These bacterial cells were heated at 75°C for 30 min to obtain heat-killed *L. acidophilus* TISTR 450 (HK-LA450), heat-killed *L. casei* subsp. *rhamnosus* TISTR 047 (HK-LC047), heat-killed *Lactobacillus* sp., LCC1 (HK-LCC1) heat-killed *Lactobacillus* sp., LCC2 (HK-LCC2) and heat-killed *E. coli* ATCC 25922 (HK-EC).

### Cell lines

Caco-2 cells were obtained from the Faculty of Pharmaceutical Sciences, Ubon Ratchathani University. The cells were cultured in Dulbecco's modified Eagle's minimal essential medium (DMEM; Gibco™, Invitrogen corporation, USA) supplemented with 10% heat-inactivated (30 min, 56°C) fetal calf serum, 2 mM L-glutamine, 100 Uml<sup>-1</sup> penicillin and 100 mgml<sup>-1</sup> streptomycin at 37°C in an atmosphere of 5% CO<sub>2</sub> 95% air.

### Adhesion assay

For adhesion assay, Caco-2 monolayers which were modified from Morita et al. (2002), Murosaki et al. (1998) and Tuomola and Salminen (1998), were prepared in 25 cm<sup>2</sup> tissue culture flasks. Cells were subcultured every 2 days. Monolayer cells were seeded up to 80% confluence, gram stained with trypan blue and counted. Adjusted cell to final concentration 1 x 10<sup>4</sup> cells ml<sup>-1</sup> to obtain confluence used in adhesion assays.

The adherence of heat-killed bacterial strains to Caco-2 cell cultures was examined by adding HK-LA450, HK-LC047, HK-LCC1, HK-LCC2 and HK-EC into well with concentration 10<sup>8</sup> cfu ml<sup>-1</sup>. After incubation, the monolayers were washed three times with sterile Triton X-100, fixed with methanol, gram stained, and examined microscopically. The numbers of heat-killed bacteria attached to Caco-2 cells was counted. Each sample was conducted in triplicate and each time was assessed in 20 randomly chosen microscopic fields. The adhesion ratio (%) was calculated as follows:

$$\% \text{ Adhesion} = \left( \frac{\text{average h2}}{\text{average h0}} \right) \times 100$$

Where, average h2 and average h0 were the amount of the cell at 2 h and initial time, respectively.

### Cytokine-Induction of heat-killed lactobacilli and HK-EC

Cytokine assay was modified from Murosaki et al. (1998), Morita et al. (2002) and Sashihara et al. (2006). Briefly, Caco-2 cells were seeded on sterile 24 well flat-bottom plates at a concentration of 1 x 10<sup>4</sup> cells ml<sup>-1</sup>. Then, all heat-killed tested strains (10<sup>8</sup> cfu ml<sup>-1</sup>) were added and incubated at 37°C in an atmosphere of 5% CO<sub>2</sub> 95% air. After 24 h incubation, the cell culture supernatants were centrifuged in order to remove cells.

**Table 1.** Specific growth rate, number of cells and percent adhesion of HK-LA450, HK-LC047, HK-LCC1, HK-LCC2 and HK-EC to Caco-2 cells.

Strain	Specific growth rate: $\mu$ ( $\text{h}^{-1}$ )	Number of bacterial cells ( $\text{Log cfu ml}^{-1}$ ) at $\text{OD}_{600} = 0.5$	Percent adhesion, mean $\pm$ SD
<i>L. acidophilus</i> TISTR 450	0.75 <sup>A</sup>	9.92 $\pm$ 0.24 <sup>A</sup>	6.68 $\pm$ 0.49 <sup>C</sup>
<i>L. casei</i> sub. <i>rhamnosus</i> TISTR 047	0.77 <sup>B</sup>	8.74 $\pm$ 0.13 <sup>B</sup>	7.41 $\pm$ 0.14 <sup>b</sup>
LCC1	0.83 <sup>C</sup>	8.48 $\pm$ 0.11 <sup>C</sup>	7.95 $\pm$ 0.60 <sup>a</sup>
LCC2	0.81 <sup>D</sup>	8.52 $\pm$ 0.18 <sup>D</sup>	7.52 $\pm$ 0.05 <sup>b</sup>
<i>E. coli</i> ATCC 25922	0.87 <sup>E</sup>	8.23 $\pm$ 0.20 <sup>E</sup>	5.68 $\pm$ 0.05 <sup>d</sup>

Specific growth rate ( $\mu$ ) with different big letters indicated significant differences ( $p < 0.05$ ) between strains; number of bacterial cells ( $\text{Log cfu ml}^{-1}$ ) at  $\text{OD}_{600} = 0.5$  with different italic big letter indicated significant difference ( $p < 0.05$ ) between strains. Percent adhesion with different small letters indicated significant differences ( $p < 0.05$ ) between strains.

To analyse cytokine induction, supernatants from treated bacterial cells were collected. The concentration of the cytokines IL-6, IL-10 and IL-12 in the supernatant were determined using commercially available enzyme linked immunosorbent assay (ELISA) kit (Quantikine<sup>®</sup> IL-6, 10 and 12 Immunoassay, USA).

## RESULTS

### Heat-killed lactobacilli preparation

The growth profile of each microorganism was investigated such as optical density and growth number, which were investigated every hour (data not shown). Specific growth rate of 4 strains lactic acid bacteria and *E. coli* ATCC 25922 were obtained by growth profile study as shown in Table 1. *E. coli* ATCC 25922 had the highest specific growth rate of  $0.87 \text{ h}^{-1}$  with the number of cells ( $\text{OD}_{600} = 0.5$ )  $8.23 \pm 0.20 \text{ cfu ml}^{-1}$  or had the lowest generation time. *L. acidophilus* TISTR 450 had the lowest specific growth rate of  $0.75 \text{ h}^{-1}$  with the number of cells ( $\text{OD}_{600} = 0.5$ )  $9.22 \pm 0.24 \text{ cfu ml}^{-1}$  or highest generation time. Furthermore, specific growth rate was significantly different in all group ( $p < 0.05$ ).

### Adhesion assay

The percent adhesion to Caco-2 cells varied among the tested bacteria in strain-dependent manner ( $p < 0.05$ ) (Table 1). The percentage of adhesion was between  $5.68 \pm 0.05$  and  $7.95 \pm 0.60\%$ . HK-LCC1 was the most adhesive strain in this study since approximately  $7.95 \pm 0.60\%$  of the added bacteria bound to Caco-2 cell cultures. HK-EC was the least adhesive strain. However, the adhesion of HK-LCC2 did not significantly differ from HK-LC047. Nevertheless, all heat-killed lactobacilli adhered better than HK-EC.

### Cytokine-stimulatory activities

The ability of the immune cells associated with the Caco-2 to respond to different heat-killed lactobacilli was

assessed by the cytokine release. We first examined the ability of the HK-LA450, HK-LC047, HK-LCC1, HK-LCC2 and HK-EC to induce IL-6, IL-10 and IL-12. Table 2 showed the productions of IL-6, IL-10 and IL-12, which were  $3.17 \pm 0.36$  to  $4.63 \pm 0.7$ ,  $143.81 \pm 0.63$  to  $255.59 \pm 1.22$  and ND to  $2.45 \pm 0.11 \text{ pg/ml}$ , respectively. All heat-killed lactobacilli strongly enhanced IL-10 when compared to HK-EC. HK-LCC1 induced the highest IL-10 production in Caco-2. Heat-killed lactobacilli did not stimulate IL-12 production. Whereas, HK-EC induced IL-12 production.

## DISCUSSION

*L. acidophilus* TISTR 450 and *L. casei* sub. *rhamnosus* TISTR 047 were used as standard strains because of well-known properties. LCC1 and LCC2 were isolated from fermented plant beverages (FPBs) from our previous experiment. *E. coli* ATCC 25922 was used as a control because it is a Gram-negative bacteria and some strains of *E. coli* are pathogen. Among all *Lactobacilli* tested strains, LCC1 had the highest specific growth rate ( $\text{h}^{-1}$ ). Bacterial cell counts are higher in the short time. The number of cells and specific growth rate is used to evaluate the time of cultured bacteria for preparation of dead cell. Growth profile was used to calculate the cell number of bacteria before process to dead cell.

Bacterial adhesion is considered important as one of the selection criteria for probiotic strains. So, the Caco-2 adhesion was important for this study. HK-LCC1 had the best adhesion property when compared with standard strains. Besides, HK-LCC2 adhered as well as HK-LC047, which is the standard strain. The adhesion of heat-killed lactobacilli may be caused by several mechanisms mediated by adhesin (Beachey, 1981; Busscher and Weerkamp, 1987); (2) the carbohydrates on the bacterial cell wall appeared to be partly responsible for the interaction between the bacteria and the extracellular adhesion-promoting factor (Coconnier et al., 1992). Furthermore, Schillinger et al. (2005) reported

**Table 2.** Production of cytokines by Caco-2 when stimulated by HK-LA450, HK-LC047, HK-LCC1, HK-LCC2 and HK-EC.

Strain	IL-6 (pg/ml)	IL-10 (pg/ml)	IL-12 (pg/ml)
<i>L. acidophilus</i> TISTR 450	3.17 ± 0.36 <sup>c</sup>	171.33 ± 1.28 <sup>C</sup>	ND
<i>L. casei</i> sub. <i>rhamnosus</i> TISTR 047	4.03 ± 0.49 <sup>b</sup>	147.04 ± 0.63 <sup>D</sup>	ND
LCC1	4.63 ± 0.70 <sup>a</sup>	255.59 ± 1.22 <sup>A</sup>	ND
LCC2	3.86 ± 0.37 <sup>b</sup>	241.77 ± 1.56 <sup>B</sup>	ND
<i>E. coli</i> ATCC 25922	3.83 ± 0.41 <sup>b</sup>	143.81 ± 0.63 <sup>E</sup>	2.45 ± 0.11

Data are shown as means ± SD of triplicate experiments; ND = not detect; means of individual trials within a column with different small letters (IL-6) and big letters (IL-10) are significantly different ( $p < 0.05$ ) with each column.

that the hydrophobic potential of strains differed considerably. Hydrophobicity plays a key role in first contact between a bacterial cell and mucus or epithelial cells. Correlation between percent adhesion and cytokine induction of heat-killed lactobacilli was not found in this study (Pearson's correlation level  $r = 0.39$ ,  $P = 0.01$ ). Palencia et al. (2008) found that bacteria adhered to the Caco-2 cell line with the highest adhesions being observed. Binding of all strains to Caco-2 cell did not result in significant increase in the production of cytokines.

Intestinal inflammation is one potential target for probiotic therapy; clinical improvement and protective effects have been shown in food allergies and atopic dermatitis with probiotic (Kalliomaki et al., 2001). Cytokine induction is the determinant important probiotic activity; for example, immune modulation, pathogen exclusion, enhanced healing of damaged mucosa and prolonged transient colonization (Alander et al., 1999); it is thought to be important for modulating the immune system (O'Halloran et al., 1998). The proinflammatory cytokines are secreted by the epithelium, such as TNF- $\alpha$ , IL-1, IL-6, IL-8 and IL-12 (Isolauri, 1999). The inhibition of proinflammatory cytokines and the supplementation of anti-inflammatory cytokines reduced inflammation. For instance, IL-12 is a proinflammatory cytokine and a modulator of cell-mediated immunity, which is mainly produced by macrophage, dendritic and B cell. Lipopolysaccharide (LPS) from gram negative bacteria induced the production of IL-12. IL-6 is a pro-inflammatory and anti-inflammatory cytokine. It is secreted by T cell and macrophage. LPS induces IL-6 in BEAS-2B and A549 cells (Schulz et al., 2002). IL-10 is a cytokine that regulates immune-mediated inflammation. It appears to have two major functions: To inhibit cytokine (that is, TNF, IL-1, chemokine and IL-12) production by macrophages and inhibit the accessory functions of macrophages in T cell activation. The effects of these actions cause IL-10 to play mainly an anti-inflammatory role in the immune system. Bacterial DNA and cell walls, such as capsular polysaccharides, lipoteichoic acids, peptidoglycan (PGN) and LPS can stimulate Caco-2 cells to produce IL-6 and IL-10. Nevertheless, the heat-killing method used in this study denatured DNA. Therefore,

bacterial DNA did not take into account the IL-10 induction in this experiment. In this study, we were interested in the study of the production of IL-6, IL-10 and IL-12. It was found that HK-LCC1 can stimulate highest IL-6 and IL-10. Besides, it was found that HK-LCC2 could stimulate IL-6 as well as HK-LC (no significant difference). All heat-killed lactobacilli could not induce IL-12, which promote chronic inflammation when compared with HK-EC, probably due to LPS present in the cell wall. Mechanism of cytokine induction by these heat-killed lactobacilli is being undertaken.

Adherence of bacteria to the epithelial intestinal cells is an important prerequisite for colonization by microorganisms and virulence manifestations (Falkow et al., 1992). Inhibiting the adhesion of pathogenic bacteria to their receptor could decrease the intestinal colonization and in consequence modify the process of pathogenicity. The immune inducer of probiotics may also partially explain their protective *in vivo* effect.

In conclusion, the present work demonstrated that several heat-killed lactobacilli are potent inducers of different cytokines on Caco-2. Production of IL-6 and IL-10 is also induced by heat-killed lactobacilli. Heat-killed lactobacilli are more potent IL-10 inducer than HK-EC. Induction of proinflammatory cytokines IL-6 and anti-inflammation cytokine IL-10 by heat-killed lactobacilli could indicate that heat-killed lactobacilli can stimulate nonspecific immune responses. Hence, heat-killed lactobacilli might prevent or reduce inflammatory responses. We could use heat-killed lactobacilli as living microorganism as probiotics for the purpose of stimulating nonspecific immunity. Application of heat-killed lactobacilli will have a beneficial effect as physiologically functional foods with much stability than using live cell *Lactobacillus*.

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