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Aggregation and adhesion abilities to enterocyte-like HCT-116 cells of probiotic candidates *Lactobacillus plantarum* strains isolated from "mandai", Indonesian fermented food against enteropathogens

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Abstract

Ten probiotic candidates of *L. plantarum* strains isolated from "mandai" were evaluated for aggregation, adhesion to HCT-116 cells and competition abilities towards enteropathogens (*Listeria monocytogenes* ATCC 13932, enteropathogenic *Escherichia coli* (EPEC) K1.1 and *Salmonella enterica serovar* Typhimurium ATCC 14028). The results showed that four *L. plantarum* strains had good autoaggregation ability above 50%, but only two strains demonstrated coaggregation ability (above 25%) with all enteropathogens tested. Five strains of *L. plantarum* showed the ability to adhere to HCT-116 cells above 60%, with the highest adhesion (74%) was performed by MB427 after 1 h incubation at 37°C. *L. plantarum* strains showed stronger competitive inhibition, exclusion and displacement towards EPEC than *S.* Typhimurium and *L. monocytogenes*. *L. plantarum* MB427 strain showed strong inhibition abilities towards the three pathogens except in displacing of *L. monocytogenes*. These results suggest that *L. plantarum* strains isolated from "mandai" were promising as probiotic candidates acquired protection against certain enteropathogenic infection.

Introduction

The development of probiotic foods especially with a specific health benefit has drawn much attention to the scientist. The characteristics of the probiotic strain incorporated into the food product will be very important before the food itself can be claimed as a probiotic food. FAO/WHO (2002) defined probiotics as live microorganisms which when administered in adequate amounts confer a health benefit to the host. Probiotics have been reported having some abilities to protect human host from gastrointestinal infections. Some probiotics exerted benefits such as prevention of necrotizing enterocolitis, antibiotic associated diarrhoea and acute infectious diarrhea. Although in healthy person, probiotic effects were not always consistent, however, probiotics can provide consistent beneficial effect in specific immunocompromised or health challenge person (Culligan *et al*., 2009, Bron *et al*., 2012).

Previous studies (Lee *et al*., 2003, Collado *et al*., 2007, Lim *et al*., 2008) reported that the health benefit especially in the prevention of gut infection exerted by the probiotics was strain-specific. Therefore, studies have been done to obtain the specific strain isolated from many sources, including fermented food products. "Mandai" is a popular fermented product in East Borneo, Indonesia. Mandai is a spontaneous lactic acid fermentation of the outer part of fruit flesh of . Lactic acid bacteria (LAB) isolates had been collected from the previous study (Emmawati *et al*., 2015) during mandai fermentation and characterized for probiotic profiles. Ten LAB isolates having good probiotic potency had been screened and identified as *Lactobacillus plantarum*. In order to develop probiotic foods using these potential probiotic candidates, further evaluation should be done to evaluate the ability of the probiotic candidates in preventing gut infection by enteric pathogens such as *Listeria monocytogenes*, EPEC and *Salmonella* Typhimurium.

Probiotics could contribute to human health according to some mode of actions. Probiotics may inhibit pathogenic bacteria by competitive exclusion, either by direct inhibitory or displacement activity. Some probiotics strains may modulate signaling pathway to enhance epithelial barrier function that lead to enhance production of some proteins that regulate defence system. Probiotic may modulate the immune system of the host. The adherence ability

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in the intestine of the host is required to exert the beneficial health effect of probiotic strains (Candela *et al*., 2008, Ouwehand *et al*., 2002).

Bacterial adhesion was initially based on non-specific physical interaction between two surfaces, adhesin and the receptor. Adhesion to intestinal epithelial cells is an important prerequisite for colonization of probiotic strains in the gastrointestinal tract, preventing the elimination by peristalsis and providing a competitive advantage in this ecosystem. The ability of *Bifidobacterium* strains (Gueimonde *et al.*, 2007) and *L. delbrueckii* subsp *bulgaricus* (Abedi *et al*., 2013) were reported to inhibit the adhesion and to displace selected pathogens from human intestinal mucus. The ability of probiotic bacteria to compete pathogens for adhesion sites on the intestinal mucosal surface needs more investigation (Collado *et al*., 2006).

Autoaggregation of probiotic strains appeared to be necessary for adhesion to intestinal epithelial cell and coaggregation abilities may form a barrier that prevents colonization by pathogenic microorganisms (Del Re *et al*., 2000, Collado *et al*., 2007a). The autoaggregation and adhesion abilities of probiotic strains were mediated by proteinaceous components on cell surface, such as S-layer protein (Kos *et al*., 2003). Coaggregation ability of a probiotic strains with pathogens correlated with autoaggregation and hydrophobicity ability can be used to indicate adhesion and competition ability of a probiotic strain with pathogens (Collado *et al*., 2007a).

Adhesion ability to the gastrointestinal mucosa was influenced by the microbiota and the probiotics involved. To allow pathogens to colonize and infect the host mucosa epithel, adherence to intestinal epithel was required. Pathogen inhibition by probiotic could provide protection against gastrointestinal infection. Protection mechanism against pathogens included competition of binding site and nutrient uptake. The adhesion and the competition against pathogens were specific for each probiotics and for each pathogen (Lee *et al.*, 2003, Collado *et al.*, 2007a, Collado *et al*., 2007b). Combination of some strains of probiotics could enhance the exclusion of pathogens adhesion compared to a single strain (Collado *et al.*, 2006).

The aims of this research were to to study the aggregation, adherence and competition abilities of *L. plantarum* strains isolated from "mandai" in order to evaluate their potency to inhibit the enteropathogens (*Salmonella enterica serovar* Typhimurium, enteropathogenic *Escherichia coli* (EPEC) and *Listeria monocytogenes*) infections in the gut.

Materials and Methods

Bacterial strains and growth conditions

Ten *L. plantarum* strains were obtained from the previous study (Emmawati *et al*., 2015) and grown in Man-Rogosa-Sharpe (MRS) broth at 37°C for 48 h. Enteric bacterial cultures, *Salmonella enterica serovar* Typhimurium ATCC 14028 and *Listeria monocytogenes* ATCC 13932 were grown in Brain Heart Infusion (BHI) broth. Enteropathogenic *Escherichia coli* (EPEC) K1.1 was obtained from Biology Department, Bogor Agricultural University and cultured in Triptic Soy Broth (TSB) supplemented with 100 µg/ml ampicillin. Two other pathogens used in the study were *Enterococcus faecalis* ATCC 19433 and *Bacillus cereus* ATCC 10876 were grown in MRS broth medium and BHI broth medium, respectively.

Autoaggregation assay

Autoaggregation assays were performed according to Kos *et al.* (2003) with slight modifications. The *L. plantarum* strains were grown for 18 h at 37°C in MRS broth. The cells were harvested by centrifugation at 5000 g for 10 minutes, washed twice and resuspended in phosphate buffered saline (PBS) in certain volume to give viable counts of approximately 10^8 CFU ml^{-1}. Four ml of cell suspensions were mixed by vortexing for 10 s and autoaggregation was determined after 4 h of incubation at room temperature. About 0.1 ml of the upper suspension was transferred to another tube with 3.9 ml of PBS and the absorbance (A) was measured at 600 nm. The autoaggregation percentage is expressed as: $1-(A_t/A_0) \times 100$, where A_t represents the absorbance at time t=4 h and A_0 the absorbance at t=0.

Coaggregation assay

The *L. plantarum* strains and the five enteropathogens mentioned above were grown for 18 h at 37°C with MRS broth and BHI broth, respectively. About 2 ml of each cell suspension were mixed in pairs. Control tubes contained 4 ml of each bacterial suspension on its own. The absorbance (A) at 600 nm of the suspensions was measured after 4 h of incubation at room temperature. Samples were taken in the same way as in the autoaggregation assay. The percentage of coaggregation was calculated using the equation of Handley *et al.* (1987):

$$\text{Coaggregation (\%)} = (((A_x+A_y)/2)-A_{x+y}) / (A_x+A_y) \times 100$$

where x and y represent each of the two strains in the control tubes, and (x + y) represent the mixture of both strains.

Adhesion assay to cell lineculture

The assay was performed as a modification of Pan *et al.* (2009) to study the adhesion abilities of the best five *L. plantarum* strains selected based on the autoaggregation and coaggregation abilities and the adhesion abilities of three enteropathogens to enterocyte-like cells. The effects of the probiotic strains and the pathogens interaction with enterocyte-like cells including competitive inhibition, exclusion and displacement were also determined. Culture of HCT-116 cell (ATCC CCL 116) as a simulation of enterocyte-like cells were used in this assay. Three enteropathogens used in this adhesion assay were *Salmonella enterica serovar* Typhimurium ATCC 14028, *Listeria monocytogenes* ATCC 13932 and Enteropathogenic *Escherichia coli* (EPEC) K1.1.

The 18 h *L. plantarum* strains in MRS broth and pathogens in BHI broth were separately harvested by centrifugation and washed twice with PBS. Bacterial cells were diluted in antibiotic-free Dulbecco's Modified Eagle Medium (DMEM) (pH 7.3, 25°C) and 0.5 ml/well of the bacterial suspension (10^7 CFU ml^{-1} for pathogenic bacteria and 10^8 CFU ml^{-1} for lactic acid bacteria) was added to 24-well tissue culture plates. Plates were incubated for 1 h at 37°C and washed three times with sterile PBS. The cell-associated pathogens (extracellular plus intracellular bacteria) were lysed with 1% (v/v) Triton X-100 (Sigma) in deionized water for 5 min. This concentration of Triton X-100 did not affect bacterial viability for at least 30 min (Pan *et al*., 2009). Serial dilution were performed and cells were plated on Palcam Agar medium for *L. monocytogenes*, Xylose Lysine Dextrose (XLD) agar medium for *S. enterica serovar* Typhimurium and Levine-Eosin Methylene Blue (EMB) Agar medium for EPEC. Adhesion was calculated as the percentage of bacterial counts recovered after adhesion relative to the number of the bacterial suspension added to the mucus.

Competition assay

Preparation of the bacterial cultures and the assay were performed as described above. The ability of lactobacilli to inhibit enteric pathogen bacteria adhesion (10^7 CFU per well) to HCT-116 cells was evaluated by simultaneous addition of 10^8 CFU per well of tested *L. plantarum* strains followed by 1 h incubation at 37°C. The assay was determined in three independent experiments, and each assay was performed in triplicate to calculate intra-assay variation. The competition was calculated as the difference between the adhesion of the pathogen in the absence and presence of probiotic.

Displacement assay

Preparation of the bacterial cultures and the assay were performed as described above. The ability of *L. plantarum* strains to displace enteric pathogen bacteria adhesion (10^7 CFU per well) to HCT-116 cells after 1 h incubation at 37°C was evaluated by addition of 10^8 CFU per well of tested lactobacilli isolates followed by 1 h incubation at 37°C. Appropriate dilutions of the lysate were plated on appropriate media as previous. Displacement of pathogens was calculated as the difference between the adhesion after the addition of the probiotic and the corresponding control buffer.

Exclusion assay

Preparation of the bacterial cultures and the assay were performed as described above. The ability of 10^8 CFU per well of tested lactobacilli to defence the exclusion site, after 1 h incubation at 37°C, against pathogenic strains adhesion (10^7 CFU per well) to HCT-116 cells was evaluated by addition of pathogen followed by 1 h incubation at 37°C. Appropriate dilutions of the lysate were plated on appropriate media as previous. Competitive exclusion was calculated as the percentage of pathogens bound after the combination with probiotic relative to pathogens bound in the absence of probiotic.

Statistical analysis

The data values were presented as mean of standard of deviation of triplicate measurements. Data were subjected to one-way ANOVA and continued by Duncan test to identify the differences in the assay. Differences was considered as statistically significant if $P< 0.05$.

Results

Autoaggregation and coaggregation ability of L. plantarum *strains*

Aggregation is a phenotype related to cell adherence properties. Among the ten strains of *L. plantarum*, only four strains i.e. MC805, MC807, MB411 and MB427 exhibited good autoaggregation abilities above 50%, with the highest autoaggregation was shown by *L. plantarum* MB427 strain (Figure 1). According to Koss *et al.* (2003), autoaggregation could be related to the cell surface component, because it was not detached after washing and suspending the cells in PBS.

Coaggregation abilities of *L. plantarum* strains isolated from mandai with five enteropathogens were also evaluated. Data are expressed as the percentage reduction after 4 h in the absorbance of a mixed

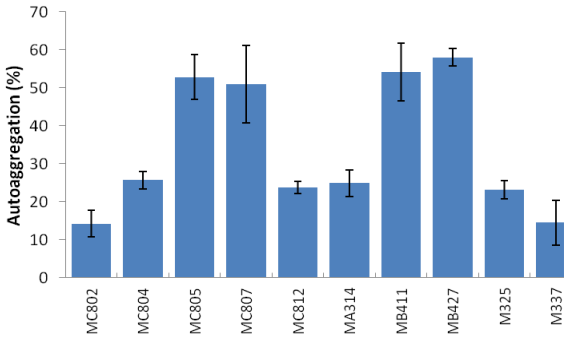

Figure 1. Autoaggregation index of *L. plantarum* strains from mandai

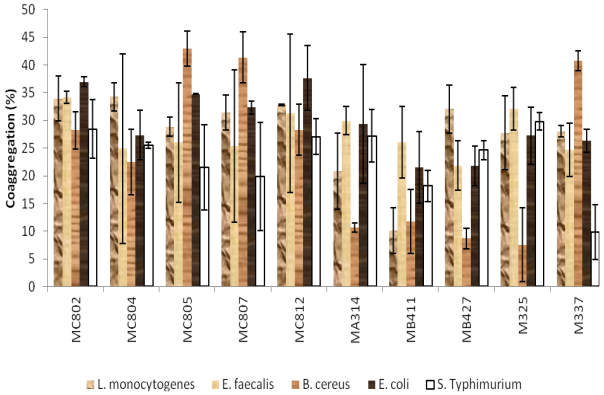

Figure 2. Coaggregation index of *L. plantarum* strains from mandai on five enteric pathogens

suspension compared with the individual suspension. In general, all *Lactobacillus plantarum* strains could be able to coaggregate with five enteropathogens tested, but the degree of abilities were found to be specific, depending on the strains used (Figure 2). Only two *L. plantarum* strains (MC802 and MC812) demonstrated good coaggregation abilities (above 25%) with all five enteropathogens. The best coaggregation abilities (above 30%) summarized as follows, were shown by *L. plantarum*MC 802, MC804, MC807, MC812 and MB427 strains towards *L. monocytogenes*; *L. plantarum* MC802, MC812, and M325 strains towards *E. faecalis*; *L. plantarum* MC805, MC807, and M337 strains towards *B. cereus*; *L. plantarum* MC802, MC805, MC807, and MC812 strains towards *E. coli*. The best coaggregation ability (above 25%) towards *S.* Typhimurium were performed by *L. plantarum* strains i.e. MC802, MC804, MA314 and M325.

Four *L. plantarum* strains (MB427, MB411, MC807, MC 805) which exhibited the highest autoaggregation ability and one strain (MC802) exhibited with relatively good coaggregation abilities (above 25%) with all pathogens tested were chosen for further examination of adhesion to enterocyte-like HCT-116 cells.

Adhesion to enterocyte-like HCT-116 cells

The five *L. plantarum* strains showed quite good adhesion ability above 60% to HCT-116 cells. The highest ability was observed in MB427 strain with 74% adhesion to HCT-116 cells after 1 h incubation at 37°C (Figure 3). The adhesion abilities of the three pathogens varied. *S.* Typhimurium showed the highest adhesion ability (62%), followed by EPEC K1.1 (52%) and *L. monocytogenes*(51%).

Competition, exclusion and displacement assay

Competition for adhesion to HCT-116 cells was observed when the *L. plantarum* strain and enteropathogenic bacteria were incubated together. In the exclusion study, the *L. plantarum* strains were allowed to adhere to the HCT-116 cells first and then the enteropathogenic bacteria was added subsequently, in contrast with the displacement study.

In general, the ability to compete with, exclude, and displace of enteropathogenic bacteria adhesion to HCT-116 cells varied between *L. plantarum* strains. Similarly, the degree of competition, exclusion and displacement of enteropathogenic bacteria by *L. plantarum* strains were also varied between species (Table 1). The data demonstrated that each *L. plantarum* strain could only compete with a limited range of enteropathogenic bacteria for adhesion sites. Degrees of displacement, competition and exclusion were specific for each strain of *L. plantarum* and for each enteric bacterium. The strongest competitive inhibition, exclusion and displacement of adhesion of enteropathogenic bacteria by the *L. plantarum* strains were shown towards EPEC. Two strains with the highest ability to compete with, exclude and displace of EPEC were MB411 and MB427. The *L. plantarum* MC802, MC805 and MB427 strains were observed having the highest ability to compete with, exclude and displace towards *S.* Typhimurium. The *L. plantarum* MB427 strain exhibited the strongest abilities to compete with, exclude and displace towards the three pathogens, except in displacing of *L. monocytogenes*.

The strongest ability of *L. plantarum* strains to compete with the three enteropathogens was observed towards EPEC (21.99-41.70%), followed by *S.* Typhimurium (13.30-24.07%) and *L. monocytogenes* (13.21-25.38%). *L. plantarum* MB427 strain exhibited the highest competition ability towards all three pathogens.

All *L. plantarum* strains were able to exclude all three enteropathogens, however the degree of exclusion abilities were strain-dependent. The best exclusion ability was shown towards EPEC (20.52-

Table 1. Adhesion abilities to HCT-116 cells of *L. plantarum* strains against enteropathogens

Enteropathogens	*L. plantarum* strains	Adhesion abilities of the strains (%)		
		Competitive exclusion	Inhibition	Displacement
L. monocytogenes	MC802	13.21 ± 3.49^{c}	12.22 ± 1.92^{b}	4.77 ± 3.49^{c}
	MC805	25.38 ± 1.88^{a}	5.27 ± 1.44^{b}	15.33 ± 0.82^{a}
	MC807	17.21 ± 1.92^{bc}	21.45 ± 5.46^{a}	6.89 ± 0.82^{bc}
	MB411	22.87 ± 4.52^{ab}	23.65 ± 2.95^{a}	10.20 ± 1.08^{b}
	MB427	22.66 ± 1.65^{ab}	29.42 ± 1.04^{a}	6.33 ± 0.78^{c}
EPEC K1.1	MC802	21.99 ± 0.85^{c}	30.57 ± 3.82^{a}	37.94 ± 1.66^{a}
	MC805	32.25 ± 0.77^{b}	20.52 ± 0.47^{b}	25.88 ± 2.08^{b}
	MC807	29.53 ± 2.03^{b}	21.34 ± 0.10^{b}	23.01 ± 1.74^{b}
	MB411	41.70 ± 4.08^{a}	30.11 ± 2.57^{a}	32.62 ± 1.32^{a}
	MB427	39.17 ± 2.79^{a}	26.86 ± 5.86^{ab}	34.86 ± 4.57^{a}
S. Typhimurium	MC802	24.07 ± 1.03^{a}	22.50 ± 1.77^{a}	26.23 ± 2.15^{a}
	MC805	22.71 ± 1.50^{a}	21.71 ± 7.03^{a}	22.10 ± 2.85^{ab}
	MC807	13.30 ± 5.50^{b}	12.36 ± 0.11^{b}	17.67 ± 4.08^{b}
	MB411	14.71 ± 0.67^{b}	21.98 ± 2.07^{a}	25.37 ± 1.57^{a}
	MB427	22.50 ± 1.18^{a}	17.29 ± 0.52^{ab}	23.37 ± 1.28^{ab}

Means within the same column with different superscript are significantly different ($P<0.05$)

30.57%), followed by *S.* Typhimurium (12.36-22.50%) and *L. monocytogenes* (5.27-29.42%). *L. plantarum* MB411 and MB427 strains showed the highest ability in excluding the three enteropathogens tested.

All pathogens can be displaced by *L. plantarum* strains with the degree of displacement varied among strains. The highest displacement was observed towards EPEC (23.01-37.94%), followed by *S.* Typhimurium (17.67-26.23%) and *L. monocytogenes* (4.77-15.33%). Among *L. plantarum* strains, MC802 was the best strain in displacing EPEC and *S.* Typhimurium but poor in displacing *L. monocytogenes.*

Discussion

Ten potential probiotic *L. plantarum* strains isolated from "mandai" were examined for their autoaggregation and coaggregation ability with five enteropathogenic bacteria. Aggregation of bacterial cells is related to cell adherence abilities (Kos *et al.*, 2003). All *L. plantarum* strains showed autoaggregating ability above 10%, four strains among them exhibited autoaggregation ability above 40%. According to Del Re *et al*. (2000), strains with autoaggregation ability below 10% suggested as nonautoaggregation.

It was indicated that autoaggregation ability related to cell surface components and there was no extracellular component involved in the autoaggregation. However, Abdulla *et al*. (2014) observed the significant decrease in autoaggregation ability of *Lactobacillus* strains which was resuspended in PBS compared with those resuspended in MRS broth.

Coaggregation assay was performed to observe interbacterial adherence, between *L. plantarum* strains and some enteropathogenic bacteria. Coaggregation abilities of the *L. plantarum* strains with 5 enteropathogenic bacteria were varied dependent to the strain. The strongest coaggregation ability with all enteropathogens tested was observed in MC802 and MC812, with value above 25%. Similar coaggregation abilities of *L. plantarum* strains were reported by Dias *et al*. (2013). The average percentage of coaggregation activity of 32 *L. plantarum* strains with three enteropathogenic, *L. monocytogenes, E. coli* and *S.* Typhi, were above 25%. The probiotic strains should show the ability to coaggregate with the enteropathogenic strains tested but the degree of coaggregation is strain-specific (Collado *et al*., 2007). Coaggregation abilities of the *L. plantarum* strains with enteropathogenic bacteria might prevent colonization of the gastrointestinal tract by the enteropathogenic bacteria and contribute as important host defense mechanism (Kos *et al.*, 2003).

Autoaggregation and coaggregation assay in the present study were performed for 4 h.According to Dias *et al.* (2013), the autoaggregation of the *L. plantarum* strains increased linearly over time from 0-4 h, but after 4 h the increasing of autoaggregation was not significant. Cell adhesion is a multi step process involving contact of the bacterial cell membrane and interacting surfaces. Aggregation ability is related

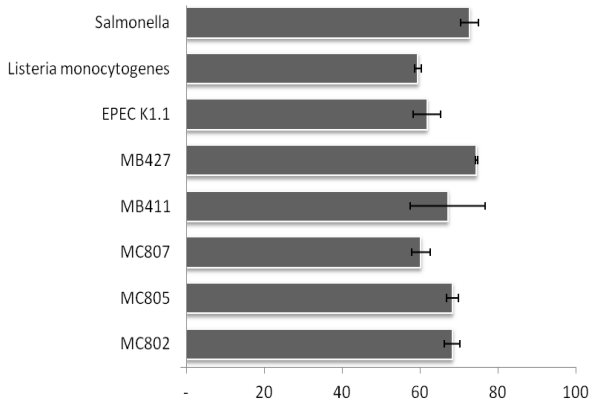

Figure 3. Adherence (%) of *L. plantarum* from mandai and three enteric pathogens to HCT-116 cell

to cell adherence properties (Del Re *et al*., 2000). These results indicate a high potential ability of *L. plantarum* strains isolated from "mandai"to adhere to epithelial cells and mucosal surfaces. The ability has been suggested as an important property of many bacterial strains used as probiotics (Bao *et al*., 2010).

The five *L. plantarum* strains show high adhesion ability to HCT-116 cells as well as three enteropathogenic bacteria with adherence of more than 50% (Figure 3). The adhesion of *L. plantarum* strains showed variability depending on the strain ranging from 60% to about 75%. The present study indicated that there is a positive correlation between the adhesion ability to HCT-116 cells with autoaggregation ability, which were shown by the five selected *L. plantarum* strains. In general, adhesion abilities of *L. plantarum* strains were higher than the three enteropathogens. The high adhesion ability indicated strong capability to bind to the intestinal mucus and possibility to colonize the intestinal cells. Lower adhesion ability was reported by Collado *et al.* (2006a, 2006b, 2007) showing the adhesion ability of less than 20% for the probiotic strains and below 15% for enteropathogenic strains. HCT-116, human colon carcinoma cells have been used by other reseachers to evaluate probiotic and pathogens adhesion to intestinal epithel (Wang *et al.*, 2008).

The probiotic dosage was important for optimizing adhesion to intestinal cells. Piatek *et al.* (2012) reported that increasing in dose of probiotic used for adhesion resulted in the increasing of number of adhering bacteria to intestinal cells. In this research, we used dose of 1 x 10^8 CFU/ml *L. plantarum* strain added to HCT-116 cells. This number was considered to be sufficient to demonstrate their adhering adhesion abilities.

The competition with, exclusion and displacement of enteropathogenic bacteria by *L. plantarum* strains were highly strain-dependent. The strongest competition, exclusion and displacement abilities of several *L. plantarum* strains in this study were observed towards EPEC, followed by *S.* Typhimurium and *L. monocytogenes*. Collado *et al.* (2007) reported some probiotic strains, one of them was *L. plantarum*, were not able to compete with, exclude and displace of *E. coli, L. monocytogenes* and *S. Typhimurium*. However, in combination, some probiotic were able to compete with, exclude and displace of *E. coli, L. monocytogenes* and *S.* Typhimurium.

Selection of probiotics being able to compete directly with pathogens would be a logical approach to the selection and development of probiotics for therapeutic treatment of gastrointestinal infectious diseases (Lee *et al*., 2003). To allow LAB isolates for autoaggregation and adherence, S-layer proteins could be considered important. Bacterial cells that had been extracted with 5 mol l^{-1} LiCl to remove S-layer protein showed reduction of adherence (Kos *et al.*, 2003). For *L. plantarum*, the ability to adhere to intestinal cells was reported involved mannose specific adhesion to bind mannose-containing receptor sites in epithelial cells (Gross *et al*., 2010).

Conclusions

The present results confirm that adhesion ability of lactic acid bacteria as well as their ability to compete with, exclude and displace enteropathogenic bacteriawas strain dependent. All ten *L. plantarum* strains isolated from "mandai" showed autoaggregating ability above 10% with the highest autoaggreation ability (above 40%) showed by four strains. The strongest coaggregation ability with all enteropathogenic tested was observed in strains MC802 and MC812, with value above 25%. Five *L. plantarum* strains showed high adhesion ability to HCT-116 cells, as well as three enteropathogenic bacteria, however, *L. plantarum* strains showed higher adhesion ability as compared to the pathogenic bacteria. This suggests that each specific *L. plantarum* strain would be able to colonize the intestinal cells and compete with a specific enteropathogen. The strongest competition, exclusion and displacement ability of *L. plantarum* strains were observed towards EPEC, followed by *S.* Typhimurium and *L. monocytogenes*. From the overall results, *L. plantarum* MB427 strain can be considered as the best performance strain that may play an important role in preventing of gut infection by three enteropathogens studied. However, this health benefit needs to be confirmed further by *in-vivo* study.

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