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Original Article

Potential probiotic evaluation of two *Lactobacillus plantarum* strains isolated from Indonesian fermented food and fruit

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Abstract

Two Lactobacillus plantarum strains, InaCC B122 and InaCC B128, isolated from Indonesian fermented food, "dadih" and fruit were evaluated for probiotic potential. These two lactobacilli strains were subjected to a series of in vitro test to assess their surviving capability in gastrointestinal track and antimicrobial activity. The test included acid and bile salt tolerance, adhesion to the yeast *Saccharomyces cereviseae* cells, heat resistance and antimicrobial activity against *Mycobacterium smegmatis*, *Escherichia coli*, and *Staphylococcus aureus*. Both strains were able to grow under acid and bile salt condition for 24 h. Co-aggregation percentage of strain InaCC B122 and InaCC B128 against the yeast cells was 52.3 % and 53.8 % respectively. Heat treatments (45 ℃ for 30 min. and 55 ℃ for 15 min.) did not affect co-aggregation percentages. Their supernatants had antimicrobial activity against *M. smegmatis*, *E. coli* and *S. aureus* with MIC of 5 %, 10 % and 10 % respectively. In conclusion, based on this assessment, these strains are good candidate for probiotic.

Keywords: acid tolerance, antimicrobial, bile salt tolerance, cell adhesion, *Lactobacillus plantarum*, probiotic

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Introduction

Probiotics defined as live microorganisms that if administered in adequate amounts can confer a health benefit to the host (FAO & WHO, 2011). The majority of probiotics are bacteria especially lactic acid bacteria (LAB) including genera *Lactobacillus* and *Bifidobacterium*. The LAB are generally used in many fermented foods such as fermented milk, cheese, and vegetable-based foods. Nowadays, the gastrointestinal health benefit linked to the probiotics consumption including the reduction of diarrhea and the improvement of bowel syndrome, lactose intolerance, antibacterial properties, and also have beneficial on controlling diabetes and reducing blood glucose (Lye *et al.*, 2009).

Lactobacillus plantarum is a member of the facultatively heterofermentative group of lactobacilli that are frequently isolated from plant material and various fermented foods (Vesscovo *et al* , 1993). *L. plantarum* is one of the most predominant *Lactobacillus* species in human intestinal bacteria (Arhne *et al.*, 1998). This species has ability to adapt to a variety environmental condition and have been shown to effectively survive to the GI tract. Some research also reported that *L. plantarum* can persist in the intestine of healthy volunteers for up to 11 days after cessation (Goossens *et al.*, 2003). This information have led to the selection of *L. plantarum* strain for probiotic properties and claimed to be functional food components that provide beneficial effect to the host (Molin, 2001).

The probiotic bacteria have role to offer benefits to the host, so probiotic must overcome physical and chemical barrier. Its means, probiotics must tolerate the acid and bile in the GI tract (Del Piano *et al.*, 2006). In mammals, the use of LAB for probiotics due to their properties of antibacterial activity agains pathogens, immunomodulation, control of homeostasis, gastric acidity resistance, and bile resistence. Blum *et al* (1999) state that to select a new microbial strain for potential probiotics use, the strain must be screened for their transit tolerance to simulated GI track condition, including pH and bile salt. These microbe must have strong ability to adhere to intestinal epithelium, which is a prerequisite for improving intestinal microbiota to exert beneficial health effect. Intestinal adhesion of probiotic bacteria can be done with using *S. cereviseae* as simulation model, because of their specific adhesin that reported for *L. plantarum* which binds with mannose residues as present in the intestinal cell line (Zago *et al.*, 2011).

L. plantarum strains InaCC B122 and InaCC B128 were LAB strains were isolated from *Dadih* (fermented milk) and Strawberry fruit repectively. There were no information about potency of probiotic. The objective of this study was therefore to evaluate probiotic potential of two strains of *L. plantarum* from Indonesia based on acid and bile resistance, adhesion to the yeast cell and antimicrobial activity. *E. coli*, *S. aureus* and *M. smegmatis* was used for antimicrobial activity. *M. smegmatis* is non-pathogenic mycobacterium and used as an attractive model organism and a surrogate host for genetic analysis of the worldwide pathogen *Mycobacterium tuberculosis* (Jacobs, 2000).

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Method

Strain and Culture Condition

Lactobacillus plantarum strains were obtained from Indonesian Culture Collection (InaCC). *L. plantarum*

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strain InaCC B122 and InaCC B128 were isolated from *Dadih* (fermented milk) and Strawberry fruit, repectively. The strains were inoculated on MRS (de Man, Rogosa, and Sharp, SIGMA) broth. *Saccharomyces cereviseae* strain InaCC Y93 were obtained from Indonesian Culture Collection (InaCC) and used for co-aggregation assay to simulate initial adhesion. This strain was inoculated on ME (Malt Extract) medium and incubated at room temperature during 24 h without shaking (Ferrando *et al.*, 2016). Three microorganisms, *Escherichia coli* strain K12, *Staphylococcus aureus* and *Mycobacterium smegmatis* were used for antimicrobial assay and inoculated on NB (nutrient broth, HIMEDIA) medium.

Acid Tolerance Assay

The effect of low pH on the survival of *Lactobacilli* was examined by inoculating 20 µL of sample culture to the 180 µL of MRS medium on sterile flat-bottom 96-well micro plate. The strains were grown on MRS broth ajusted to pH 2; 2,5; 3.5; 7 and 8 and incubated at 37 ℃ for 24 h (Agaliya *et al.*, 2012). Changes in optical density were measured by using micro plate reader at 595 nm following 0; 0.5; 1; 1.5; 2; 3; 4; 5; 6; 7; and 24 h of incubation at 37 ℃ (Jacobsen *et al.*, 1999).

Bile Tolerance Assay

The effect of bile salt on the survival of *Lactobacilli* was examined by inoculating 20 µL of sample culture to the 180 µL of MRS medium on sterile flat-bottom 96-well micro plate. The strain were grown on MRS broth containing 0.05; 0.1; 0.3; 0.6 and 1 % bile salt (SIGMA) and incubated at 37 ℃ for 24 h (Agaliya and Jeevaratnam, 2012). Changes in optical density were measured by using micro plate reader at 595 nm following 0; 0.5; 1; 1.5; 2; 3; 4; 5; 6; 7; and 24 h of incubation at 37 ℃ (Jacobsen *et al.*, 1999).

Co-Aggregation with *Saccharomyces cereviseae* after heat treatment

L. plantarum strains InaCC B122 and InaCC B128 were grown on MRS broth pH 7. Heat treatments were applied as follows: heat adaptation (45 ℃ for 30 min) and heat shock (55 ℃ for 15 min). After treatment, the culture was centrifuged at 2270 g 15 ℃ for 15 min, washed with PBS (pH 7.4). Then the pellet was suspended in PBS buffer (0,1 M pH 7.4), concentrating 5 times to initial volume. The suspension was centrifuged at 3500 rpm 15 ℃ for 15 min, washed twice in PBS buffer and suspended in the same buffer to final concentration of 1 % (w/v). The co-aggregation mix consisted of 2 ml of yeast suspension, 1 ml of *L. plantarum* suspension and 1 ml of PBS buffer. The control was mixtured by 2 ml of yeast suspension and 2 ml PBS. The mixes were shaken for 20 min and incubated at room temperature for 1 h. After incubation, 10 ml of supernatant were taken and counted the yeast by using haemocytometer. Co-aggregation percentages were calculated using formula:

$$\% \text{ Co-aggregation} = [1-(Nf/Nc)] \times 100$$

While Nf is the final yeast count in the mixture and, Nc is the final yeast count in the control supernatant (Ferrando *et al.*, 2016).

Antimicrobial Activity Assay

Antimicrobial activity was determined by observing minimum inhibition concentration (MIC) of LAB supernatants, using MTT (Thiazolyl Blue Tetrazolium Blue) assay (Cui *et al.*, 2013). Cell free supernatants were obtained by separating media from 48 h LAB cultures by centrifugation at 3500 *g*, 15 min. Suspensions of *M. smegmatis*, or *E. coli*, or *S. aureus* was inoculated into 100 µl NB media containing supernatan with a serie of concentration (40 %, 20 %, 10 %, 5 %, 2,5 % and 0 %, v/v) in 96 wells microplate. The initial concentration of suspensions was 10^{-2} of that of preculture in stationary phase. The cultures were then incubated in room temperature and shaking with 100 rpm. After 72 h (*M. smegmatis*) or 24 h (*E.coli* and *S. aueus*) cultures were added with 10 µl of MTT solution (5 mg/ml) and incubated for 2 h and added with 11 µl propanol containing 0, 04 M HCl, incubated for 1 h . Viable cultures reduce MTT (yellow) into formazan (purple) wich is observed by microplate reader at λ 595 nm. MIC was defined at concentration where OD value was equal to that of media. Experiments were carried out in triplicate.

Results

Acid and Bile Tolerance

Growths of both LAB strains on media with several value pH of 2.5; 3.5, 7, and 8 are presented in figure 1. The growth curves of both strains indicated that *L. plantarum* InaCC B122 and InaCC B128 were able to grow well under acid condition for 24 hours. The data strongly showed that *L. plantarum* InaCC B122 and InaCC B128 were accessed as acid-tolerance strains.

The growths of both LAB strains in media containing 0.05 %; 0.1 %; 0.3 %; 0.6 % and 1 % of bile salt concentration are presented in figure 2. Compared with control (0 %), the growths of LAB were similar for difference concentration of bile salt. The data indicated *L. plantarum* B122 and B128 are able to grow in media containing 1 % bile salt for 24 h.

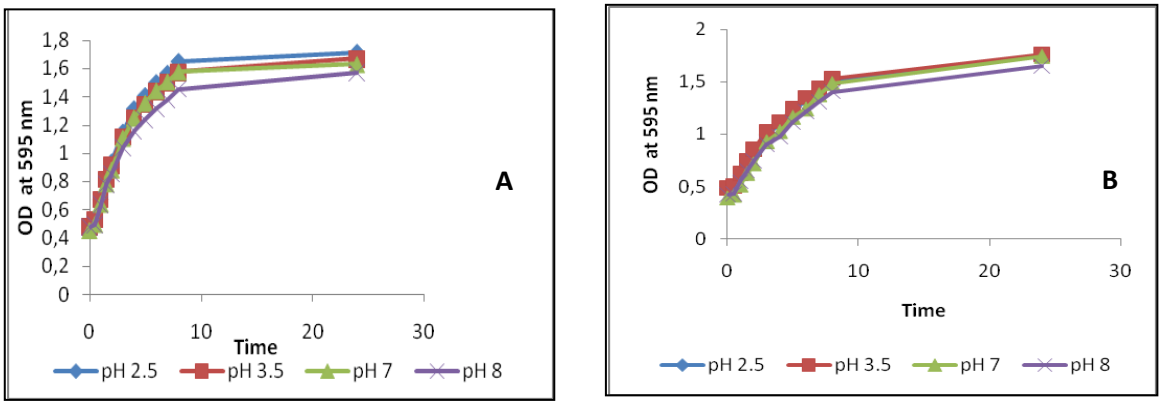

Figure 1. *L. plantarum* growth under various pH. (a) *L. plantarum* InaCC B122 and (b*) L. plantarum* InaCC B128

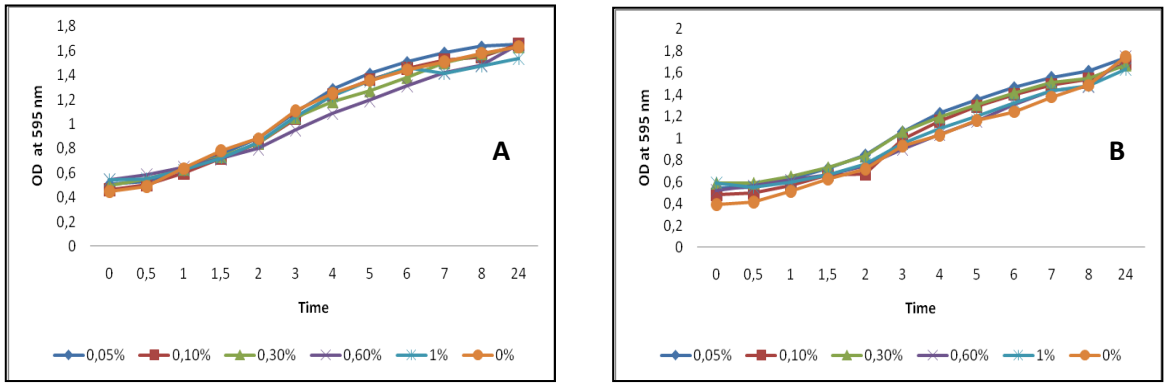

Figure 2. *L. plantarum* growth under various bile concentration. (a) *L. plantarum* InaCC B122 and (b*) L. plantarum* InaCC B128

Co-Aggregation with *S. cereviseae* after heat treatment

Co-agregation percentages (% Co) of *L. plantarum* InaCC B122 and InaCC B128 with *S. cerevisiae* were similar for the three conditio (control, 45 °C, and 55 °C) (Figure 3). This is based on the statistical analysis using ANOVA (α= 0.05), showing no significant differences between both of *L. plantarum* strains neither in each condition nor for each strain in three conditions.

Antimicrobial Activity

Supernatans of *L. plantarum* InaCC B122 and InaCC B128 were able to inhibit the growth of *M. smegmatis*, *E. coli* and *S. aureus* with MIC of 5 %, 10 % and 10 % respectively (Table 1).

Table 1. Antibacterial activity of the supernatants of LAB

LAB strain	MIC of LAB supernatant against microbial target (%, v/v)		
	M. smegmatis	*E. coli*	*S. aureus*
L. plantarum InaCC B122	5	10	10
L. plantarum InaCC B128	5	10	10

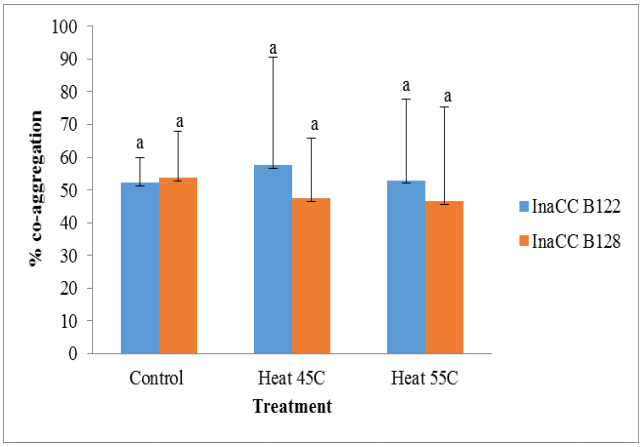

Figure 3. Co-aggregation percentage of *L. plantarum* with *S. cereviseae* in control condition and heat treatment.

Discussions

Some important characteristics of good probiotic are their resistance to low pH and bile salt, ability to adhere epithelium cell, and also resistance to unexpected environment during mass production such as high

temperature (Ferrando *et al*., 2016). We evaluated such probiotic properties in Indonesian LAB, *L. plantarum* InaCC B122 and InaCC B128.

We revealed that the two strains are resistant to acidity. Acids is found in the human stomach. This substance may disturb the biomolecules of cells, such as fatty acid, protein and DNA. Low pH environment can inhibit the metabolism and reduce growth and viability of bacteria (Chan *et al.*, 2011). The GI tract or stomach has low pH, its about 2,5-3,5. This in an effective barier preventing bacteria from entering the intestinal track. Generally, food travels through the stomach for 2-4 hours (Huang & Adams, 2004). The ability of LAB strains InaCC B122 and InaCC B128 to grow in acid condition indicated that they will survive living in GI tract. Another study showed that 29 *Lactobacilli* strains from dairy for probiotic properties were able to survive at pH 3.0 (Maragkoudakis *et al.*, 2006). Previous work has demonstrated that the acid tolerance of bacteria was related to the induction of H^+-ATPase activity (Matsumoto *et al.*, 2004).

Bile salts are harmful to living cells, because its can damage the cell membrane structure. The average of bile salt in human intestine is about 0.3 % (w/v). Therefore, the tolerance to the bile salt of strains was believed to be an essential property to select new probiotic strains, that can survive in the intestine (Succi *et al.*, 2005). The evaluation of bile tolerance of bacteria for probiotic properties can be meassured by growth abilities of the isolated strains in their culture media (Mandall *et al.*, 2006). In this study, we showed that those strains could grow in media containing 1 % bile salt. Mandall *et al* (2006) reported that, after bacterial exposure to the bile salt, there are disruptions of cellular homeostasis and caused the dissociation of billayer lipid and integral protein of the cell membrane. This condition caused leakage of the bacterial content and cell death. So, the bacteria that survive on the bile condition is a good probiotic candidates. The resistance of bacterial to the bile salt could be due to bile salt hydrolase activity (BSH) that deconjugates bile salts and inactivates its powerful antimicrobial action (Van de Guchte *et al.*, 2002). Probiotic bacteria must resist the action of pancreatic enzymes that have effect on the cell wall or membrane component that affecting their viability. *L. plantarum* InaCC B122 and InaCC B128 have ability to grow under bile salt concentration.

To select microbial strain for probiotic properties, the strains must be able to adhere to the intestinal epithelium cell (Blum *et al.*, 1999). Co-aggregation with yeast is superficial properties related with the adhesion to intestinal epi-thelium cells. Yeast cell that use for co-aggregation assay is *S. cereviseae* that was proposed by Alderberth *et al* (1996). *S. cereviseae* has specific adhesin that reported for *L. plantarum* which bind with mannose residues as present in intestinal cell lines such as HT29, so that this cell used as an in vitro model to simulate intestinal adhesion (Zago *et al.*, 2011). The capacity of bacteria especially probiotic bacteria to adhere to the intestinal cell has been associated to high auto-agregation and hydrophobicity values (Servin & Cocconier, 2002). Both of *L. plantarum* InaCC B122 and InaCC B128 have ability to adhere to *S. cereviseae* with % co-aggregation 52,3 % and 53,8 % respectively. Another *L. plantarum* strain Lp 813 and Lp 998 have high co-aggregation with *S. cereviseae* with the value 77 % and 68 % (Ferrando *et al.*, 2016). Heat treatment that used for this studies does not give signifficant effect to co-aggregation properties of *L. plantarum* InaCC B122 and InaCC B128. Other studies reported that there are no differences of *L. plantarum* 83114 and *L. kefir* 8321 adhesion to Caco-2 cells before and after spray drying, as a result of thermal, osmotic, and oxidative stress treatment (Golowczyc *et al.*, 2011). Servin and Cocconier (2003), also reported that there is an efficient adhesion of *L. acidophilus* adhesion to Caco-2 cell before and after lethat heat treatment.

Based on the antibacterial activity of LAB, there are several strains among *Lactobacilli* bacteria with probiotic properties have more application in prevent infection. Their inhibitory action due to production of lactic acid, bacteriocins and deacetyl (Hassanzadazar *et al.*, 2012). Strus *et al* (2001), also reported that some bacteria produce peptides that having inhibitory properties. Most of lactic acid bacteria produce bacteriocins, it is a peptide with bactericidal activity against strains of closely related species. This bacteriocins may prevent the growth of harmful bacteria in the fermentation and preserve food product (Lahtinen *et al.*, 2004).

Based on the explanation above, its can be concluded that *L. plantarum* strain InaCC B122 and InaCC B128 are good probiotic candidates. Both of these species are able to growth under acid and bile salt condition. They also exhibited antimicrobial activity against *E. coli* and *S. aureus* with MIC 10 % and *M. smegmatis* with MIC 5 %, respectively. Both of *L. plantarum* strain InaCC B122 and InaCC B128 have ability to adhere to intestinal epithelium which using *S. cereviseae* as simulation model in this study. There is no significant differences between control and heat treatment to the capabilities of both species to adhere to the yeast cell.

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