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Antimicrobial activity and adhesion ability of indigenous lactic acid bacteria isolated from goat milk

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Abstract

To be categorized as probiotic bacteria, lactic acid bacteria (LAB) must have at least antimicrobial activity and adhesion ability on intestinal mucosal surfaces. The aim of this study was to determine the antimicrobial activity and adhesion ability of eight LAB strains isolated from goat milk. The isolates passed initial selection on low pH (2.0, 2.5, and 3.0) and bile salt tolerance (0.3% Oxgall). Three strains of *L. rhamnosus* (TW2, TW3, TW32); five strains of *L. plantarum* (TW4, TW10, TW14, TW26, and TW28) were examined for their antimicrobial activity against both spoilage and pathogenic bacteria (*S.* Typhimurium ATCC 14028, *E. coli* ATCC 8739, *B. cereus* ATCC 13061, *S. aureus* ATCC 25923 and *P. aeruginosa* ATCC 9027). Five isolates (TW2, TW4, TW14, TW28, and TW32) were evaluated for their ability to adhere to intestinal mucosal surfaces. A modified well diffusion method was used to assess the antimicrobial activity. A modification of an animal model was also used to analyze the LAB adhesion ability. The adhesion of the bacteria on jejunum and ileum was examined by using Scanning Electron Microscope. The result showed that the isolates had diameters of inhibition ranging from 12.6 to 19.9 mm for *S.* Typhimurium, 11.3 to 21.4 mm for *E. coli*, 7.5 to 19.9 mm for *B. cereus*, and 9.9 to 24.7 mm for *P. aeruginosa*. *L. plantarum* TW10 and TW26 had no inhibition activity for *S. aureus*. The adhesion ability of the bacteria was 0.54-2.19 log CFU/cm^2 on the intestinal mucosal surfaces. The highest adhesive level in jejunum and ileum was showed by *L. rhamnosus* TW2.

Introduction

According to FAO/WHO (2002), antimicrobial activity and adhesion ability on intestinal mucosa are two requirements for bacteria to be categorized as probiotic. Lactic Acid Bacteria (LAB) with antimicrobial activity could act as inhibitor of spoilage and pathogenic microorganisms in food through their antagonistic properties. Some strains are also known to have preservation properties by producing organic acids, diacetyl, hydrogen peroxide (Oyetayo *et al*., 2003) or bacteriocin (Ten-Brink *et al.*, 1994).

In addition to antimicrobial activity against pathogens, adhesion ability is also important and is associated with stimulation of immune system and colonization. In the case of LAB, it is a complex process which begins with the contact to cell membrane. This important interaction is needed to prevent elimination process due to peristaltic movement of the intestine. Some *in vitro* researches using a solid surface such as stainless steel-plate (Evanikastri, 2003), caco-2 cell culture (Collado, 2006), and rat's epithelial cells (Anggraeni, 2010) to evaluate the adhesion ability of LAB on intestine. Kos *et al.* (2003) studied the adhesion of *L. acidophilus* M 92 to swine's ileum microscopically and showed that it was able to attach firmly to the ileum.

As many as 16 LAB isolates had been isolated by Setyawardani *et al.* (2011) from goat milk of crossbreed Etawa (PE) and 17 others from crossbreed Saanen (PESA). From these 33 isolates, eight isolates (2, 3, 4, 10, 14, 26, 28, and 32) were able to survive at 0.3% bile salt solution which continued with a decrease in the number of microbes as much as 1-2 logs. Three of the eight isolates showed resistance to low pH condition (2.0, 2.5, and 3.2). The study used API test to identify the microbes which were potential to be probiotic LAB. The result showed that *L. rhamnosus* TW2, TW3, and TW32; *L. plantarum* TW4; and *L. plantarum* TW10, TW14, TW26, and TW28 were identified as potential probiotic LAB.

The aim of this study was to perform an *in vitro* selection of the eight isolates based on the antimicrobial activity and adhesion ability to the rat's

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intestinal mucosa.

Materials and Methods

The LAB used were *L. rhamnosus* TW2, TW3, and TW32; *L. plantarum* TW4; and *L. plantarum* TW10, TW14, TW26, and TW28 isolated form goat milk. The *in vitro* antimicrobial activity was performed by using well diffusion method; using medium such as Mueller-Hinton Agar (MHA) from Merck, de Man Rogosa Sharpe Agar (MRSA) from Difco, de Man Rogosa Sharpe Broth (MRSB) from Difco, and Tryptocase Soy Broth (TSB) from Difco; and several American Type Culture Collection (ATCC) pathogenic bacteria which were obtained from the culture collection of Department of Food Science and Technology, Bogor Agricultural University, i.e. *Salmonella* Typhimurium (ATCC 1408), *Bacillus cereus* (ATCC 13061), *Staphylococcus aureus* (ATCC 25923), *Escherichia coli* (ATCC 8739) and *Pseudomonas aeruginosa* (ATCC 9027).

The adhesion ability was determined by using duodenum, jejunum, ileum, and cecum of 2 months old male Sprague Dawley (SD) rats, MRSB medium, and Phosphate Buffered Saline (PBS) solution. The equipment used in this study was micropipette, threaded test tubes, petri dishes, autoclave, centrifuge, vortex, refrigerator, digital scale, freeze drier, and a set of Scanning Electron Microscope/SEM (JSM-5310LV JEUL Japan).

Antimicrobial activity of indigenous lactic acid bacteria isolated from goat milk

The test used well diffusion method based on Liasi *et al.* (2009) with modification in concentration. Firstly, bacterial culture of *S.* Typhimurium, *B. cereus*, *S. aureus*, *E. coli*, and *P. aeruginosa* were maintained and stored at 5°C. The cultures were refreshed every week. Dilution of the stock culture was made to achieve a population of 10^6 CFU/mL. A total of 1 mL diluted culture was poured onto a petri dish and added with 20 mL of 40°C MHA (Savadogo *et al*., 2004). The medium was allowed to harden and holes 5 mm in diameter were made. Secondly, a sample of 2 mL was concentrated by using freeze drier, and then 50 µL of the bacterial culture was introduced into the hole and kept at 8°C for 30 minutes. The culture was incubated at 37°C for 24 hours. The inhibition zone which was a clear area around the well was measured by using a caliper for three times in three different places and the result was averaged. The test was measured for three times in duplicate replications (duplo).

Adhesion ability of indigenous lactic acid bacteria isolated from goat milk in rat's intestine

The rat's intestine was opened and washed with PBS solution three times until clean (Mayra-Maukinen *et al*., 1983). Bacterial cultures were grown for 24 hours in MRSB medium, and then separated by centrifugation at 5000 rpm for 15 minutes (Nitisinprasert *et al*., 2006). The obtained precipitate was washed with physiological solution and then suspended to reach 8 log CFU/mL. A total of 10 mL of the LAB suspension was poured onto a petri dish containing rat's intestine-cut slices and incubated for 60 minutes (Anggraeni, 2010) at room temperature (30°C). Afterward, the intestine was rinsed with PBS twice. The total number of LAB was counted by using total plate count method. Furthermore, the data was processed statistically with SPSS 17.0 software, including one way Analysis of Variance (ANOVA) and continued with Duncan test. Besides of using total plate count method, Scanning Electron Microscopy was also used to observe the adhesion image of LAB in the intestine.

Adhesion observation of indigenous lactic acid bacteria isolated from goat milk in rat's intestine

A modification method from Ali *et al.* (2009) was made in the dehydration stage of the SEM method. The method started with placing the intestine samples into a solution of glutaraldehyde 4% w/v in phosphate buffer (pH 7.2, 40°C) for 12 hours, and then washed with 0.1 M cacodylate buffer three times for 10 minutes. The next step was the dehydration process which was carried out by using gradual concentration of ethanol (50, 70, 85, 95, and 100% v/v). The cells then was dried by freeze drying and coated with gold to observe the adhesion of LAB to the intestine with electron microscope.

Results

Antimicrobial activity of LAB

This study indicated that all of the LAB strains had antimicrobial activity as shown by the obtained diameter of inhibition toward: a) *S.* Typhimurium (12.6 – 19.9 mm), b) *E. coli* (11.3 – 21.4 mm), c) *B. cereus* (7.5 – 19.9 mm), and d) *P. aeruginosa* (9.9 – 24.7 mm). However, some strains did not show any antimicrobial activity toward *S. aureus* such as *L. plantarum* TW10 and TW26, showed by no existence of clear zones. Some strains which could inhibit *S. aureus* for example *L. rhamnosus* TW2, TW3, and TW32; *L. plantarum* TW 4; and *L. plantarum* TW14

Table 1. Diameter of LAB inhibition toward some pathogenic bacteria

LAB Isolates	*S. aureus* ATCC 25923	*E. coli* ATCC 8739	*S.* Typhimurium ATCC 1408	*B. cereus* ATCC 13061	*P. aeruginosa* ATCC 9027
	mm				
TW 2	13.27 ± 2.9^{b}	21.4 ± 0.4^{b}	19.8 ± 2.0^{d}	16.17 ± 1.3^{d}	21.07 ± 0.5^{bc}
TW 3	10.43 ± 1.2^{b}	17.43 ± 1.3^{b}	16.43 ± 1.3^{bcd}	17.22 ± 4.4^{bc}	18.9 ± 0.9^{b}
TW 4	14.63 ± 2.0^{b}	21.4 ± 1.7^{b}	19.93 ± 1.3^{d}	19.17 ± 4.3^{cd}	20.97 ± 0.0^{bc}
TW 10	0 ± 0.0^{a}	13.63 ± 1.9^{a}	12.57 ± 1.4^{a}	7.47 ± 1.4^{ab}	9.9 ± 2.5^{a}
TW 14	14.77 ± 4.7^{b}	20.2 ± 1.1^{b}	18.6 ± 3.2^{cd}	19.67 ± 5.2^{cd}	24.7 ± 1.4^{c}
TW 26	0 ± 0.0^{a}	11.27 ± 2.7^{a}	13.27 ± 1.6^{ab}	8.33 ± 1.6^{a}	12.3 ± 3.1^{a}
TW 28	14.5 ± 0.4^{b}	19.87 ± 1.5^{b}	17.57 ± 2.6^{cd}	19.9 ± 4.5^{cd}	23.23 ± 1.6^{c}
TW 32	13.13 ± 2.0^{b}	18.7 ± 4.2^{b}	15.53 ± 1.0^{abc}	17 ± 3.2^{bc}	21.33 ± 1.5^{bc}

Different letters (a. b. c. d) in the same column show a significant difference at the significance level of 5 % ($p < 0.05$).

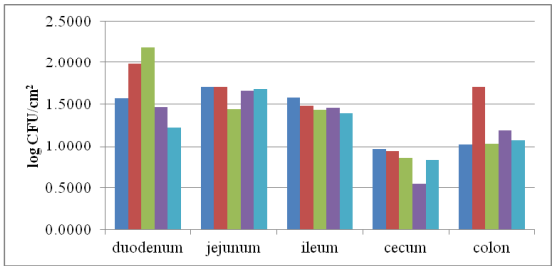

Figure 1. Increasing LAB population on intestine

L. rhamnosus TW2 ( ), *L. plantarum* TW4 ( ), *L. plantarum* TW14 ( ), *L. plantarum* TW28 ( ), and *L. rhamnosus* TW32 ( )

and TW28 showed the inhibition range between 10.4 - 14.8 mm (Table 1).

Adhesion ability of LAB to the intestine

a. Total number of adhered microbes

L. rhamnosus TW2 and TW32; *L. plantarum* TW4; and *L. plantarum* TW14 and TW28 were tested for their adhesion ability. Figure 1 shows the pieces of rat's intestine which was exposed to LAB 8 log CFU/mL, which resulted in increasing number of total LAB from 0.54 to 2.19 log CFU/cm^2. This indicated the existence of exposed LAB adhesion to the intestinal surfaces. Generally, increase in total LAB in the intestine was higher than in cecum or colon. The five isolates had ability to adhere to duodenum, jejunum, and ileum which was higher and significantly different than that in the cecum and colon ($p < 0.05$). Figure 1 shows the adhesion of LAB to duodenum exposed to *L. plantarum* TW14 which had the highest increase in total LAB followed by *L. plantarum* TW4, *L. rhamnosus* TW2, *L. plantarum* TW28 and *L. rhamnosus* TW32. The adhesion of LAB to jejunum and ileum as well as in cecum which was exposed to *L. rhamnosus* TW2 had the highest increasing of total LAB, while the highest increasing of total LAB on colon showed by *L. plantarum* TW4.

b. Observation by using Electron Microscope

The microphotograph illustrates *L. rhamnosus* TW2 and *L. plantarum* TW14 adhesion to intestinal mucous surfaces of jejunum and ileum (Figure 2 and 3). The result showed that *L. rhamnosus* TW2 on jejunum and ileum had a higher adhesion compared to *L. plantarum* TW14. Supported by counting the adhesion microbiologically, the adhesion on jejunum and ileum of *L. rhamnosus* TW2 was 1.72 log CFU/cm^2 and 1.58 log CFU/cm^2, while *L. plantarum* TW14 was 1.44 log CFU/cm^2 and 1.43 log CFU/cm^2.

Discussion

The antagonistic properties of LAB were caused by the undissociated organic acid which enter the bacterial cell and dissociate within cytoplasm. This process resulted in the reduction of intracellular pH or accumulation of ionized acids which was fatal to pathogenic bacteria (Ouwehand, 1998). Generally, the antimicrobial activity of LAB is caused by the production of organic acids, hydrogen peroxide, and protein or specific protein complex compound which is called as bacteriocin.

The antimicrobial activity tested to the four pathogenic bacteria and one spoilage bacteria showed wide inhibition diameter of >18 mm (Liasi *et al*., 2009). This can be compared to the criteria of antimicrobial activity classification which is moderate (6-9 mm), strong (10-14 mm), and very strong (15-18 mm).

The antimicrobial activity of LAB against enteropathogenic bacteria such as *E. coli* O157:H7, *Listeria monocytogenes*, *Vibrio cholerae*, and *S.* Enteritidis was due to organic acid, hydrogen peroxide, and bacteriocin or inhibition of the pathogen adhesion (Bevilacqua *et al*., 2003 and Santos *et al*., 2003). The combination of several probiotic strains was able to inhibit pathogenic bacteria in the intestinal mucous more than 40% (Collado *et al*., 2006). The diameter of inhibition of the eight LAB isolates against some pathogenic and spoilage bacteria was showed in Table 1.

In general, the results showed that the inhibitory activity of LAB against Gram-negative bacteria (*S.* Typhimurium, *E. coli* and *P. aeruginosa*) was higher than that for Gram-positive bacteria (*B. cereus* and *S. aureus*). However, a different result was obtained by Anas *et al.* (2008) which stated that *Lactobacillus* strain isolated from Algerian goat's milk had a higher inhibition against Gram-positive than the Gram-negative bacteria. The diameter of inhibition against Gram-positive *S. aureus* was 15 ± 2.26 mm, *Bacillus* sp. was 11.25 ± 2.25 mm, and against Gram-negative

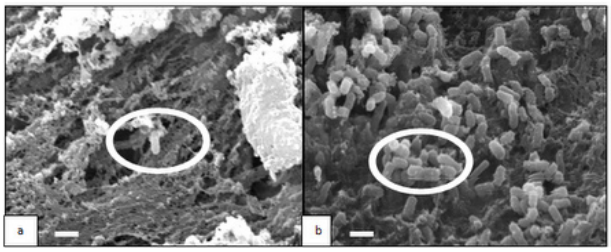

Figure 2. LAB adhesion on jejunum showed by images obtained using SEM
a. *L. plantarum* TW14 and b. L. rhamnosus TW2, 5000 x, (—) 6.6 µm

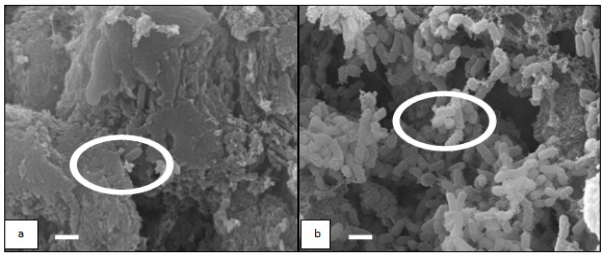

Figure 3. LAB adhesion on ileum showed by images obtained using SEM
a. *L. plantarum* TW14 and b. *L. rhamnosus* TW2, 5000 x, (—) 6.6 µm

E. coli, the diameter was 8.62 ± 1.84 mm.

Tsai *et al.* (2004) studied the antagonistic activity of LAB by neutralizing the supernatant to pH 7. Within this condition, LAB lost its antagonistic activity against the tested pathogenic strains. The antagonistic activity was originated from organic lactic acid compounds. In this study, the supernatant of *Lactobacilli* strain which was tested at pH 4.5 was able to inhibit all Gram-negative bacteria. However, there was no inhibition when the pH was raised near neutral (pH 6.5) and the inhibition was not caused by bacteriocin but organic acid (Todorov, 2008).

Studies showed antimicrobial activity of several strains such as *L. paracasei*, *L. casei* and *L. plantarum* which were able to inhibit both of Gram-positive and Gram-negative bacteria showed by the diameter of inhibition ranged from 10 to 18 mm (Liasi *et al.*, 2009). An antimicrobial activity study of *L. plantarum* and *L. rhamnosus* isolated from traditional African fermented food also showed the inhibition against *Enterococcus faecalis* (22-28 mm), *S. aureus* (16-20 mm), and *E. coli* (12-17 mm) (Kalui *et al.*, 2009).

Lactococcus showed inhibition against Gram-positive *Listeria monocytogenes* (Singh and Prakash, 2009) which ranged from 7 to 10 mm (55.5%), 10-13 mm (27.77%), and >13.3 mm (16.6%). *L. plantarum* showed inhibition against *S. aureus* (20 mm), *Bacillus* sp. (11 mm), and *E. coli* (10 mm). Therefore, *Lactobacilli* was more sensitive to inhibit Gram-positive than Gram-negative bacteria. A study analyzing antimicrobial activity of LAB in some Greek traditional cheese showed that only 6 isolates of 20 LAB strains which were *S. thermophilus* T2 had inhibition activity against Gram-positive bacteria (Mezaini *et al.*, 2009). The study continued by testing the isolates with pathogenic bacteria as the indicator. This resulted for only 5 LAB isolates which showed a very strong antimicrobial activity (>18 mm).

Similarly, Anggraeni (2010) showed an increase of 0.1-0.9 log CFU/cm^2 with 6 log CFU/mL LAB exposure. Mechanisms of adhesion to an epithelial surface involve both receptor-specific binding and charge and also hydrophobic interaction (Ljungh and Wadstrom, 2006). LAB has an ability to bind ECM (Extra Cellular Matrix) molecules such as collagen, fibronectin, and vitronectin which might be detached from epithelial cells into the layers and component of mucous.

The adhesion of LAB to the epithelial and mucous of intestine showed the onset of colonization and competition against pathogens (Martin *et al.*, 2005). *L. fermentum* PNAI, *L. reuteri* M9, and *L. marinas* PKB 1 had higher adhesion ability on mucin compared to some *Lactobacilli* strains (Olivares *et al.*, 2006). Kos *et al.* (2003) explained that the adhesion was a complex process, started from the first bacterial contact with the cell membrane and the interaction with the surfaces. Several studies had described the presence of S-layer protein forming crystals which cover the bacteria providing protection to the cells. Furthermore, protein on the cell surface was also known to be an important mediator of *L. acidophilus* M 92 adhesion.

A study on LAB adhesion ability on the intestinal mucous which was conducted by Collado *et al.* (2006) showed that all of the tested probiotic strains could adhere on the mucous. The highest percentage was the adhesion of *L. rhamnosus* GG strain by counting the differences in the amount of probiotics either in the presence of pathogen indicators or without pathogen indicator. Caco-2 *L. casei* strains which was used by Minelli *et al.* (2004) to six *L. casei* strains showed various results. Nitisinprasert *et al.* (2006) studied the adhesion efficiency by using microbiological counting method toward some types of bacteria and obtained the efficiency level from 0.89 to 21.58%.

The results of this study are consistent with Collado *et al.* (2006) which all of the LAB strains could adhere to the mucous with the highest percentage was *L. rhamnosus*. Electron microscope was able to show the adhesion on the surfaces, mucosal, and epithelial of the intestine.

Conclusion

A total of eight LAB isolates which were tested against Gram-positive and Gram-negative bacteria showed inhibition against Gram-negative. However,

L. plantarum TW10 and TW26 were not able to inhibit *S. aureus*. A total of five isolates had a good adhesion to the intestinal surfaces at levels ranged from 0.54 to 2.19 log CFU/cm^2. Adhesion of *L. rhamnosus* TW2 to jejunum and ileum were the highest as shown by increase in the number of LAB of 1.72 log CFU/cm^2 and 1.58 log CFU/cm^2, respectively. The results were supported by SEM analysis.

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