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In vivo Antimutagenicity of Dadih Probiotic Bacteria towards Trp-P1*

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ABSTRACT : *In vitro* acid- and bile-tolerant lactic acid bacteria isolated and identified from Indonesian traditional fermented milk dadih might be considered as potential probiotic strains after further characterization with animal models, especially for their therapeutic properties. Five dadih lactic bacteria isolates each had moderate survival rate for 2 h at pH 2.0, as well as bile tolerance. The aim of this research was to identify candidate probiotic lactic bacteria among indigenous dadih lactic isolates originated from Bukit Tinggi, West Sumatra, especially their *in vivo* antimutagenic property. Milk cultured with *Enterococcus faecium* IS-27526 significantly lowered fecal mutagenicity of rats as compared to the control group, skim milk, and milk cultured with *L. plantarum* IS-20506. These results suggest that *Enterococcus faecium* IS-27526 may serve as a potential probiotic strain with its antimutagenicity. (**Key Words :** Potential Probiotic, *E. faecium*, Dadih, Antimutagenicity)

INTRODUCTION

Dadih, an Indonesian traditional fermented milk of West Sumatra, is made by pouring fresh, raw, unheated buffalo milk into a bamboo tube capped with banana leaves, and allowing it to ferment spontaneously at room temperature overnight. The milk is fermented by indigenous lactic bacteria derived from the buffalo milk. Its natural fermentation provides different strains of indigenous lactic bacteria involved in each fermentation (Akuzawa and Surono, 2002).

Leuconostoc paramesenteroides was the dominant strain of lactic acid bacteria encountered in dadih originated from Bukit Tinggi (Hosono et al., 1989). In another study, Surono and Nurani found that *Lactobacillus sp*., *Lactococcus sp*. and *Leuconostoc sp*. were dominant in dadih from Bukit Tinggi.and Padang Panjang (Surono, 2004). Among the ten lactic strains from Bukit Tinggi dadih identified by the API 50CH system (Api Products, Bio Merieux, Marcy l'Etoile, France), (Torshizi et al., 2008), 5 strains were re-identified by PCR as *Lactobacillus plantarum* strains IS-10506 and IS-20506; *Enterococcus faecium* strains IS-27526, IS-23427 and IS-16183 showed potential probiotic properties with good survival rate at low pH value and in the presence of lysozyme, and short lag time in the presence of 0.3% oxgal (Surono, 2003). The competition assay, as shown by the adhesion on mucus layer between dadih LAB and selected pathogens, indicated the competence of *Enterococcus faecium* IS-16183 and *Lactobacillus plantarum* IS-10506 in significantly inhibiting the adhesion of *Escherichia coli* O157:H7, and *Enterococcus faecium* IS-27526 may inhibit the virulence of *Escherichia coli* O157:H7 and *Salmonella typhimurium* E10 by co-aggregating with the pathogens (Dharmawan et al., 2006). Collado et al. (2007) revealed that the best ability against pathogen adhesion was shown by *Lactobacillus plantarum* IS-10506. In some other studies on probiotic properties of indigenous dadih lactic bacteria, anti-mutagenic and anti-pathogenic bacteria and hypocholesterolemic properties have been proved (Hosono et al., 1990; Surono and Hosono, 1996; Surono, 2000; Pato et al., 2004).

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Carcinogenicity has always correlated with modification of gut bacterial activities. The intestinal flora comprise a complex ecosystem of a large variety of bacteria. These complex flora may produce negative and positive effects (Gorbach, 1986); altering the intestinal flora in a beneficial way may improve the health of a host. Lactic Acid Bacteria (LAB) have been considered potentially useful in this respect.

The objective of this study was to identify candidate probiotic lactic bacteria among indigenous dadih lactic isolates originated from Bukit Tinggi, West Sumatra, especially their *in vivo* antimutagenicity observed by fecal and urinary mutagenicities.

MATERIALS AND METHODS

Bacterial culture, mutagen and metabolic activator preparation

Bacterial preparation : Strains of dadih lactic bacteria were stocked in MRS broth (Oxoid, Basingstoke, England) and pre-cultured overnight in the same medium before use. Lyophilized cells were prepared by harvesting cells from MRS medium grown for 18 h. The viability and purity of the lyophilized cells was tested on MRS agar plates. Contaminants were also observed to assure that the lyophilized cells were free from contamination.

Salmonella typhimurium TA 98 and S-9 (phenobarbital- and 5,6-benzoflavon-induced golden hamster liver homogenates), a metabolic activator of rat liver, were homogenized. Both were kept in a deep freezer at -80°C until being used, and cofactor was kept in a refrigerator.

Preparation of mutagen : Amino acid pyrolyzate (Trp-P1) was used as mutagen. Two milligram of Trp-P1 (Wako Pure Chemical, Tokyo, Japan) was dissolved in 1 ml distilled water, and used for the experiment.

Metabolic activation : To test fecal mutagenicity, metabolic activation S-9 mix was prepared by using 1 ml of phenobarbital- and 5,6-benzoflavon-induced golden hamster liver homogenates (S-9, Oriental Yeast, Tokyo, Japan) added to 9 ml commercially available cofactor (Oriental Yeast, Tokyo, Japan).

Rats and diets : Twenty male Wistar rats (Japan SLC Co., Shizuoka, Japan) were obtained at eight weeks of age. The rats were fed a commercial powdered chow (Clea Japan Inc., Osaka, Japan) for two days. Animal care was in accordance with the guidelines for Animal Experimentation of the School of Medicine, Shinshu University, Matsumoto, Japan. All rats were initially adapted for 5 days to a commercially available basal chow diet (Clea Japan Inc., Osaka, Japan). After this adaptation period, rats were divided into four groups of five each. Rats were individually housed in metal cages in a room with controlled temperature (22±2°C) and humidity (56±5%) and maintained on a cycle of 12 h of light and 12 h of dark. Group 1 received commercial powdered chow plus Trp-P1 and water as a control group; group 2 received commercial powdered chow plus Trp-P1 and skim milk (SKM); group 3 received commercial powdered chow plus Trp-P1 and milk fermented with *L. plantarum* IS-20506; and group 4 received commercial powdered chow plus Trp-P1 and milk fermented with *Enterococcus faecium* IS-27526. Skim milk and fermented milks in the form of drink type, and water were freely available and the rats received their assigned diets for *ad libitum* intake for 5 d. After 3 days of preliminary feeding, 1.0 ml of Trp-P1 solution (2.0 mg/ml) mixed with diet was given to each rat for 2 d. Food intake was recorded daily and body weight was recorded at the beginning and end of the study. For the assay of fecal enzymes, fresh samples were collected at d 5 by gentle squeezing of the rectal part of rats, put into sterile test tubes in anaerobic jars and stored overnight at 4°C until analysis. For determination of fecal and urinary mutagenicity, fecal and urinary samples were collected for the last 2 days and stored at -20°C until analysis.

Preparation of skim milk and fermented milk

Skim milk (SKM) 10% (w/v) was heated by autoclave at 105°C for 5 min, each erlenmeyer containing 50 ml of the sterile skim milk was inoculated with freeze-dried cells of respective dadih LAB and incubated at 30°C until the milk coagulated. The inoculum was also added to sterile 10% SKM to produce fermented milk. Milk fermented with *L. plantarum* IS-20506 as well as with *Enterococcus faecium* IS-27526 consisted of 3.5×10^8 and 8.4×10^8 viable cells/ml, respectively. Prepared skim milk and fermented milks were kept in a refrigerator until use.

Preparation of fecal and urinary extract

Fresh faeces as well as urine of rats were collected, homogenised, 100 ml of 50:3 of 80% acetonitrile-28% ammonia solution (solution A) added to each, allowed to stand for 30 min, centrifuged at 2,000×*g* for 10 min and the supernantant collected. The same procedure was repeated, and the supernatant collected and evaporated at 35°C to dryness to obtain the extract. Then the residue was dissolved in 100 ml distilled water and 400 g of blue rayon added, shaken gently at 30°C for 30 min and the blue rayon collected. The same procedure was repeated with the addition of 300 mg blue rayon but without the addition of distilled water. The collected blue rayon was washed with 150 ml of distilled water and 100 ml of solution A was added. With the supernatant, the same procedure was repeated, and the collected supernatant was filtered using No. 2 filter paper (Advantec, Toyo, Japan), and evaporated at 35°C to dryness to collect the extract. Then 400 mg of blue rayon was added, shaken gently for 30 min, added a further 300 mg of blue rayon, shaken gently for 30 min, and

100 ml of solution A was added to the extract portion, shaken gently for 30 min, repeated twice, and again the extract was evaporated at 30°C to dryness. The final extract was dissolved in 10 ml of 10% DMSO, followed by filtration through a micro filter with a 0.45 μm pore size (Fuji Photofilm, Co., Ltd., Tokyo, Japan) aseptically to remove microorganisms. Samples were stored at -80°C until further use.

Assay for fecal enzymes

β-Glucuronidase assay : Fresh fecal samples, collected in a sterile tube and kept overnight in an anaerobic chamber (BBL, Beckton Dickinson, USA), were suspended in cold pre-reduced 0.1 M potassium phosphate buffer (pH 7.0). The fecal suspension was homogenized and was disrupted using a sonicator (Sonicator USP 300, Shimadzu, Kyoto, Japan) for 3 minutes at 4°C and then centrifuged at 500×g for 15 min, in order to remove the undissolved particles and fecal matter. High speed centrifugation was avoided as it might also have removed some enzymes and proteins. The supernatants were collected and immediately used for the enzyme assay.

The enzyme reaction was run at 37°C (pH 6.8) as described by Goldin and Gorbach (1976). Briefly, the total volume of the reaction mixture was 1 ml, containing a final concentration of 0.02 M potassium phosphate buffer, 0.1 mM EDTA, 1 mM phenolphtahelin β-D-glucuronide (Sigma-Aldrich, St. Louis, USA) and 0.1 ml fecal extract. The reaction was stopped after 15 min by adding 5 ml 0.2 M glycine buffer (pH 10.4) containing 0.2 M NaCl. Absorbance was read at 540 nm using a VERSA Max Microplate Reader (Molecular Devices, USA). The amount of released phenolphthalein was determined and compared to a standard and expressed as specific activity (μmol/mg protein/30 min). One unit of enzyme was defined as the amount of enzyme necessary to release 1 μg of phenolphthalein at 37°C in 1 min.

β-glucosidase assay : Fecal extract was prepared as described above. The enzyme reaction was run at 37°C (pH 7.4) in a total volume of 1 ml at the final concentration of 0.1 M potassium phosphate buffer, 1 mM nitrophenyl-β-D-glucoside (Sigma, USA) and 0.2 ml fecal extract. The reaction was run for 30 min at 37°C and the reaction was stopped by the addition of 5 ml of 0.01 M NaOH.

Absorbance was measured at 420 nm. The amount of nitrophenol released was determined by comparison with a standard nitrophenol curve and expressed as μmol/30 min/mg protein.

Detection of Trp-P1 in fecal and urinary samples by HPLC

Each prepared extract from fresh feces and urine which had been treated by blue rayon bound available Trp-P1 was subjected to HLPC analysis for the recovery of Trp-P1 in either fecal or urinary samples by observing remaining Trp-P1 in the samples. Analysis was carried out as described elsewhere in the *in vitro* binding assay by HPLC (Surono and Hosono, 1996).

Assay for *in vitro-in vivo* mutagenicity of fecal and urinary samples by Ames test

The same prepared extract of fresh feces and urine used for HPLC analysis was also assessed for mutagenicity by the Ames Test using *Salmonella typhimurium* TA 98 (Ames et al., 1973; Maron and Ames, 1983) to find out the mechanism of antimutagenicity.

Statistical analyses

Results obatained were analyzed by the ANOVA procedure from SPSS (Norusis, 1993), version 6.0. The least significant difference procedure was used to determine the difference of means among the treatment group.

RESULTS AND DISCUSSION

The time from entrance to release from the stomach was reported to be 90 min in human (Berrada et al., 1991), and bactericidal effect of acid is evident at pH values below 2.5 (Maffei and Nobrega, 1975). Five strains of dadih lactic bacteria, *L. plantarum* IS-10506 and IS-20506, *Enterococcus faecium* IS-27526, IS-23427 and IS-16183, had a moderate survival rate (in a range of 0.1-10%) at pH 2.0, for 2 h (Surono, 2003). Acid tolerant strains have an advantage to survive in the stomach (pH 2.0 in extreme cases) and are able to resist the digestion process in the stomach, where hydrochloric and gastric juice are secreted.

Lactobacillus plantarum strains IS-10506 and IS-20506, *Enterococcus faecium* strains IS-27526 and IS-23427 strains of dadih lactic bacteria were considered as bile-salt tolerant (du Toit et al., 1998; Surono, 2003). Bile-salt tolerance is important for strains to grow and survive in the upper small intestine, where bile salt hydrolase (BSH) activity of such lactic bacteria may play a role in the enterohepatic cycle.

There was no significant difference in β-glucuronidase and β-glucosidase activity observed among the treatment group as compared to the control (data not shown). Some fecal enzymes are considered to be indicators of carcinogenesis, in which procarcinogens are converted into carcinogens in the intestinal tract (Goldin and Gorbach, 1976). Among these, β-glucuronidase is of interest because it is the enzyme that hydrolyses biliary glucuronidases when they reach the colon (duWollowski et al., 1999). In this study, levels of both β-glucosidase and β-glucuronidase were measured in the feces of rats fed with milk fermented

Table 1. Efect of dietary milk and milk cultured with dadih lactic acid bacteria on the recovery of Trp-P1 in fecal samples of rats

Treatment group	Recovery (%)
Control	7.43[a]
SKM	5.83[ab]
FM (IS-20506)[1]	6.55[ab]
FM (IS-27526)[2]	3.81[b]

[a, b] Means in the same column followed by different superscript letter differ.
SKM = Skim milk.
[1] FM (IS-20506) = Fermented milk made from *L. plantarum* IS-20506.
[2] FM (IS-27526) = Fermented milk made from *E. faecium* IS-27526.
* $p<0.05$.

Table 2. Effect of dietary milk and milk cultured with dadih lactic acid bacteria on the recovery of Trp-P1 in urinary samples of rats

Treatment group	Recovery (%)
Control	3.94[a]
SKM	0.64[b]
FM (IS-20506)[1]	0[c]
FM (IS-27526)[2]	0[c]

[a, b, c] Means in the same column followed by different superscript letter differ.
SKM = Skim milk.
[1] FM (IS-20506) = Fermented milk made from *L. plantarum* IS-20506.
[2] FM (IS-27526) = Fermented milk made from *E.* IS-27526.
* $p<0.05$.

with *L. plantarum* IS-20506 as well as milk fermented with *E. faecium* IS-27526 strain showed no significant difference as compared to the control rat. Hence, it is reasonable to conclude that the *in vivo* antimutagenic properties of *Enterococcus faecium* IS-27526 might be due to binding ability of the bacterial cell wall of dadih lactic cultures.

When milk cultured with *Enterococcus faecium* IS-27526 was administered to the rat, it showed significant reduction of Trp-P1 as residue in the feces compared to untreated rats (Table 1). This table confirms *in vivo* antimutagenic properties of milk cultured with *Enterococcus faecium* IS-27526 dadih lactic cultures, since the recovery of Trp-P1 in fecal samples of rat fed milk cultured with *Enterococcus faecium* IS-27526 was significantly lower than other groups observed by HPLC. The mechanism involved might be due to the binding properties of bacterial cells so that recovery of Trp-P1 in fecal samples was significantly lower.

Table 2 shows that milk fermented with dadih lactic cultures *L. plantarum* IS-20506 or *E. faecium* IS-27526 and fed to the rats was able to reduce Trp-P1 to completely undetectable levels in the urinary sample, significantly lower than both the control rats and rats fed with skim milk.

Rat fed with milk cultured with *Enterococcus faecium* IS-27526 significantly decreased fecal as well as urinary mutagenicity of rats as measured by the Ames test (Tables 3 and 4). This result shows that mutagens other than Trp-P1 also could have been bound to bacterial cells which in turn lowered the fecal and urinary mutagenicity of rats. The

Table 3. Effect of dietary milk and milk cultured with dadih lactic acid bacteria on the fecal mutagenicity of rats

Treatment group	Fecal mutagenicity revertants/plate
Control	163[a]
SKM	159[b]
FM (IS-20506)[1]	142[ab]
FM (IS-27526)[2]	61[c]

[a, b, c] Means in the same column followed by different superscript letter differ.
SKM = Skim milk.
[1] FM (IS-20506) = Fermented milk made from *L. plantarum* IS-20506.
[2] FM (IS-27526) = Fermented milk made from *E. faecium* IS-27526.
* $p<0.05$.

Table 4. Effect of dietary milk and milk cultured with dadih lactic acid bacteria on the urinary mutagenicity of rats

Treatment group	Urinary mutagenicity revertants/plate
Control	158[a]
SKM	115[ab]
FM (IS-20506)[1]	100[b]
FM (IS-27526)[2]	113[ab]

[a, b] Means in the same column followed by different superscript letter differ.
SKM = Skim milk.
[1] FM (IS-20506) = Fermented milk made from *L. plantarum* IS-20506.
[2] FM (IS-27526) = Fermented milk made from *E. faecium* IS-27526.
* $p<0.05$.

mechanism of *in vivo* antimutagenicity of *E. faecium* IS-27526 was again confirmed due to the presence of *E. faecium* IS-27526 in the diet of rats. This result is in good agreement with the results of *in vitro* and some human studies which showed that consumption of lactic acid bacteria with the diet seems to reduce excretion of mutagenic activity in feces and urine (Lidbeck et al., 1992; Hayatsu and Hayatsu, 1993; Surono, 2004).

The binding of chemical mutagens with lactic acid bacteria was reported more than two decades ago, and several workers have postulated various theories on the binding of heterocyclic amines with the cell walls. It was reported to be due to cation exchange mechanisms (Morotomi and Mutai, 1986) or by the involvement of carbohydrate moieties (Hosono et al., 1990) or proteins (Tanabe et al., 1994a; 1994b).

Binding is a physical mechanism and the bound mutagens are inactive (Sreekumar and Hosono, 1998). This study provides some insight into antimutagenicity of milk cultured with *Enterococcus faecium* IS-27526.

Further work is needed to study binding mechanisms, changes in microflora composition as well as fecal and urinary mutagenicity of particular subject groups in human studies to validate *Enterococcus faecium* IS-27526 as a probiotic with antimutagenic properties. Adhesion is an important property of probiotic lactic acid bacteria able to colonize the GIT. Moreover, understanding the basic

mechanism of antimutagenic properties may have many implications for future research on probiotic lactic acid bacteria and their ability to reduce the risk of cancer.

CONCLUSIONS

Enterococcus faecium IS-27526 isolated from dadih of Bukit Tinggi, West Sumatra-Indonesia, which is an acid and bile acid tolerant isolate, exhibited *in vivo* antimutagenic properties towards Trp-P1. Rats fed milk cultured with *Enterococcus faecium* IS-27526 showed significant lower fecal mutagenicity and the recovery of Trp-P1 in urine was significantly lower than in both controls and rats fed skim milk.

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