In Vitro Probiotic Properties of Indigenous Dadih Lactic Acid Bacteria[a]

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ABSTRACT : The aim of this research was to identify candidate probiotic lactic bacteria among indigenous dadih lactic isolates. Dadih is an Indonesian traditional fermented milk of West Sumatra which is fermented naturally. Viability of the strain is critical in determining the capacity of lactic bacteria to induce immune stimulation as well as to colonize in the intestinal tract. Therefore, LAB are proposed to exert health promoting or probiotic effects in human, such as inhibition of pathogenic microflora, antimutagenic, and the reduction of cholesterol levels. This manuscript reports *in vitro* probiotic properties of indigenous dadih lactic bacteria, especially some important colonization factors in GI tract, such as lysozyme, acid and bile tolerance. Bile Salt Hydrolase (BSH) activity, spectrum of bacteriocin, and antimutagenic activity of bacterial cells were also assessed. Twenty dadih lactic isolates were screened further for their tolerance to low pH, at pH 2 and 3 as well as their bile tolerance. There were ten isolates classified as acid and bile acid tolerant, and further screened for lysozyme tolerance, BSH activity. The spectrum of bacteriocin activity of isolates was assayed using cell-free neutralized supernatants by agar spot test against variety of pathogens. *Lc. lactis* subsp. *lactis* IS-10285, IS-7386, IS-16183, IS-11857 and IS-29862, *L. brevis* IS-27560, IS-26958 and IS-23427, *Leu.mesen.mesenteroides* IS-27526, and *L. casei* IS-7257 each has good survival rate at low pH values and in the presence of lysozyme, and short lag time in the presence of 0.3 % oxgall. *Lc. lactis* subsp. *lactis* IS-11857 and IS-29862 each has high BHS activity, *Lc. lactis* subsp. *lactis* IS-10285 and IS-16183 each had a positive spectrum of bacteriocin activity against *E. coli* 3301 and *Lysteria monocytogenes* ATCC 19112, while *L. brevis* IS-26958 has high BHS activity as well as positive spectrum of bacteriocin against *E. coli* 3301, *Lysteria monocytogenes* ATCC 19112, and *S. aureus* IFO 3060. All of the ten dadih lactic strains performed *in vitro* acid and bile tolerance, indicating a possibility to reach the intestine alive, and display probiotic activities. ***(Asian-Aust. J. Anim. Sci. 2003. Vol 16, No. 5 : 726-731)***

Key Words : Duck, Mycotoxicity, Aflatoxin, Zearalenone, Fermented Chitin-chitosan

INTRODUCTION

Metchnikoff (1907) observed that the consumption of fermented milks had beneficial effect associated with the auto-digestion of lactose. Gastrointestinal microflora consist of hundreds of different types of microorganisms and are a biologically important component of the body. The effects of a species of organism in the microecology of the gut depend to some extent on the organisms' ability to survive and preferable multiply in the intestinal tract.

Lactic acid bacteria and their food products are thought to confer a variety of important nutritional and therapeutic benefits and have many documented health promoting or probiotic effects in human (Salminen et al., 1996) such as inhibition of pathogenic microflora, antimutagenic, and the reduction of cholesterol levels. Those lactic acid bacteria with scientifically supported health claims define as probiotic and have an increasingly high market potential.

Probiotics are live microbial food supplements which beneficially affect the host by improving its intestinal microbial balance. When selecting probiotics, many criteria have to be met, such as resistance to the enzymes in the oral cavity (e.g., lysozyme) and should also have the ability to resist the digestion process in the stomach and the intestinal tract. Hence, successful probiotics must have an ability to tolerate acid and bile acid.

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 allow to ferment at room temperature for two days. The milk is fermented by indigenous lactic bacteria of the buffalo milk (Surono, 2000).

Leuconostoc paramesenteroides was the dominant strain of lactic acid bacteria encountered (Hosono et al., 1989). In another study, Surono and Nurani (2001) found that *Lactobacillus sp.*, *Lactococcus sp.*, and *Leuconostoc sp.* were dominant in dadih. There have been some studies on probiotic properties of dadih indigenous lactic bacteria, such as antimutagenic, cholesterol binding and antipathogenic bacteria properties (Hosono and Tono-oka, 1995; Hosono et al., 1990; Surono and Hosono, 1996; Surono, 2000; Surono and Nurani, 2001). The aim of this research was to find out candidate probiotic lactic bacteria among the isolated indigenous dadih lactic bacteria originated from Bukit Tinggi in *in vitro* experiment.

MATERIALS AND METHODS

Bacterial preparation

Twenty colonies of lactic acid bacteria were isolated from dadih originated from Bukit Tinggi-West Sumatra,

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Indonesia, and further identified by API CH 50 test kit (BioMerieux, France), and has been identified as of *Lactococcus lactis* subsp. *lactis* (five strains), *Lactobacillus brevis* (three strains), and three strains of each *Lactobacillus plantarum, Lactobacillus casei, Lactobacillus paracasei, Leuconostoc mesen. mesenteroides* (data not shown). Pathogenic bacteria used for the bacteriocin screening were as follows: *Escherichia coli* 3301, *Listeria monocytogenes* ATCC 19112, and *Staphylococcus aureus* IFO 3060, obtained from culture collection of Technology of Animal Product, Shinshu University, Ina, Japan. *Lactobacillus casei* Rolly C, *Streptoccous thermophilus* Rolly T and *Bifidobacterium breve* Rolly B were obtained from Snow Brand Labio Milk Company, Nagoya, Japan.

Strains of dadih lactic bacteria were maintained in MRS broth (Oxoid, Basingstoke, England), and Bifidobacteria was maintained in GAM broth (Nissui, Tokyo, Japan). The pathogenic bacteria were stock in Nutrient broth (Oxoid, Basingstoke, England). All bacteria were pre-cultured over night in the respective medium at 37°C before use.

Acid and lysozyme tolerance of cultures

Among the thirteen selected acid tolerant strains which are able to grow at pH 3.5, only ten strains, *Lc. lactis* subsp. *lactis* IS-10285, IS-7386, IS-16183, IS-11857 and IS-29862, *L. brevis* IS-27560, IS 23427 and IS-26958, *Leu.mesen.mesenteroides* IS-27526, and *Lactobacillus casei* IS-7257, were found to be able to tolerate lower pH value. The effect of low pH was examined by the method of Jin et al. (1998). Each respective culture of lactic bacteria (0.1 ml) was inoculated into 5 ml of 0.05 M phosphate buffer of pH 2.0, and 3.0 adjusted with 1 N HCl, and was held at 37°C for 1 and 2 h. The colonies (cfu/ml) were counted on MRS agar.

Survival of strains in the presence of lysozyme (100 μg/ml) was determined by the cell number in 0.06 M phosphate buffer, pH 6.8 (PB) supplemented with 1 % NaCl (Heine et al., 1995). The overnight culture (0.02 ml) grown in MRS broth was inoculated into 5 ml of fresh MRS broth and incubated at 37°C for 24 h. The culture was centrifuged at 1,840×g for 15 min at 4°C. The pellet was washed twice with sterile PB and re-suspended in 5 ml of the same buffer. Lysozyme (egg white lysozyme, Wako Pure Chemical, Osaka, Japan) at 100 μg/ml was added to the culture suspension, and then the solution was held at 37°C for 60 min (Suskovic et al., 1997). A sample was taken at time zero and 60 min incubation time, serially diluted in saline and plated onto MRS plates. After incubation at 37°C overnight, the colonies (cfu/ml) were counted.

Selection of bile-tolerant strains

Bile tolerant strains were screened by the method described by Walker and Gilliland (1993). All cultures were evaluated for growth in MRS-THIO broth (MRS, Oxoid, Basingstoke, England supplemented with 0.2% (w/v) sodium thioglycolate, Nacalai Tesque, Japan), with and without 0.3% (w/v) oxgall (Difco, USA). Freshly prepared cultures were inoculated (1%) into each medium, incubated at 37°C in a water bath, and monitored hourly for growth spectrometrically at 620 nm. The growth was followed for 9 h or until a 0.3 unit difference in absorbance was reached. The effect was measured on the basis of time required to increase the absorbance at 620 nm by 0.3 units in MRS-THIO broth with and without 0.3% (w/v) oxgall. The

Table 1. Survival of lactic bacteria at low pH value

LAB*	Viable bacteria (log cfu/ml)					
	pH 3.0			pH 2.0		
	0 h	1 h	2 h	0 h	1 h	2 h
RC	8.96±0.11	6.3±0.32	5.3±0.03	9.11±0.06	5.34±0.13	5.34±0.23
RT	7.11±0.17	6.82±0.21	6.86±0.11	7.93±0.17	5.48±0.06	5.41±0.18
RB	6.6±0.04	6.92±0.13	6.9±0.21	7.49±0.11	5.49±0.23	5.33±0.06
IS-10285	8.56±0.02	7.02±0.22	6.04±0.18	7.61±0.18	5.45±0.18	5.35±0.36
IS-7386	9.2±0.05	6.87±0.14	6.22±0.26	9.22±0.16	7.77±0.16	4.83±0.09
IS-16183	8.29±0.12	7.6±0.12	4.3±0.25	7.6±0.21	5.85±0.24	5.22±0.21
IS-27560	7.15±0.23	6.75±0.16	5.76±0.31	7.8±0.16	5.41±0.22	5.15±0.06
IS-26958	7.08±0.09	4.67±0.14	4.3±0.21	7.66±0.21	5.41±0.32	5.21±0.24
IS-27526	6.78±0.13	6.67±0.21	4.67±0.18	7.69±0.34	5.48±0.14	5.35±0.31
IS-11857	8.39±0.07	7.23±0.08	6.39±0.19	7.81±0.16	5.43±0.15	5.4±0.01
IS-29862	8.17±0.09	6.47±0.15	5.98±0.22	7.46±0.12	5.19±0.26	5.42±0.27
IS-7257	8.27±0.02	7.46±0.12	4.22±0.15	7.69±0.21	5.65±0.21	5.22±0.31
IS 23427	8.11±0.19	6.73±0.11	5.66±0.25	7.55±0.12	5.16±0.20	5.49±0.22

Values are means with standard deviation of three experiments

* RC: L. casei Rolly C; RT: S. thermophilus Rolly T; RB: Bif. breve Rolly B; IS-10285: Lc. lactis subsp. lactis IS-10285; IS-7386: Lc. lactis subsp. lactis IS-7386; IS-16183: Lc. lactis subsp. lactis IS-16183; IS-27560: L. brevis IS-27560; IS-26958: L. brevis IS-26958; IS-27526: Leu.mesen.mesenteroides IS-27526; IS-11857: Lc. lactis subsp. lactis IS-11857; IS-29862: Lc. lactis subsp. lactis IS-29862; IS-7257: L. casei IS-7257; IS-23427: L. brevis IS-23427

Table 2. Bile tolerance, BSH activity and bacteriocin activity on dadih lactic cultures

LAB*	Bile tolerance[a]	BSH	Bacteriocin activity		Against ***.[b]
	0.3% (w/v) oxgall (min)	Activity**.[b]	E. coli 3301	Listeria monocyto-genes ATCC 19112	S. aureus IFO 3060
RC	22.2	+	-	-	-
RT	4.98	+	-	-	-
RB	12.36	+	-	-	-
IS-10285	0.42	+	+	+	-
IS-7386	0.9	-	-	-	-
IS-16183	0.1	+	+	+	-
IS-27560	11.82	+	-	-	-
IS-26958	11.52	++	+	++	+
IS-27526	13.8	-	-	-	-
IS-11857	16.5	++	-	-	-
IS-29862	5.03	++	-	-	-
IS-7257	10.56	-	+	+	-
IS-23427	11.2	-	-	-	-

* Refer to Table 1.

** - : BSH activity not detected; −: BHS activity detected: −+: BSH activity strongly detected.

*** - : antibacterial activity not detected: −: antibacterial activity detected: +−: antibacterial activity strongly detected.

[a] lag time: time required to increase the absorbance at 620 nm by 0.3 units in MRS-THIO broth with and without 0.3% (w/v) oxgall. The difference in time (minutes) between the culture media was considered as the lag time (LT).

[b] Confirmed in three experiments.

difference in time (minutes) between the culture media was considered as the lag time (LT).

Spectrum of bacteriocin activity

The antibacterial activity of the isolates was determined using cell-free neutralized supernatants (CFNS) obtained from cultures grown in MRS broth for 18 h at 37°C. Cultures were centrifuged and the pH of the supernatant was adjusted to 6.5 with 1 N NaOH. The supernatant was then sterilized by passing through a sterile membrane filter (0.45 µm, Advantec, Toyo, Japan), and stored in a refrigerator. The neutralized supernatants were tested against *Escherichia coli* 3301, *Listeria monocytogenes* ATCC 19112, and *Staphylococcus aureus* IFO 3060 using the agar spot test method of Uhlman et al. (1992). Clear inhibition zone of 1 mm or more will be regarded as positive inhibition.

Bile-salt hydrolytic (BHS) activity

Lactic acid bacteria isolates were screened for BSH activity by impregnating an 8 mm sterile paper disc (Advantec, Toyo, Japan) in an overnight culture, and placing the paper disc on MRS agar plates supplemented with 0.5% (w/v) sodium salt of taurodeoxycholic acid (TDCA) (Nacalai Tesque, Japan) and 0.37 g/l $CaCl_2$ (Dashkevicz and Feighner, 1989). Plates were incubated anaerobically at 37°C for 72 h, after which the diameter of the precipitation zone was measured.

Statistical analysis

Acid, bile and lysozyme tolerance are presented as mean values ±SD from triplicate samples.

RESULTS AND DISCUSSION

The time from entrance to release from stomach was reported to be 90 min (Berrada et al., 1991), and bactericidal effect of acid is evident at pH values below 2.5 (Maffei and Nobrega, 1975). The result revealed that 10 strains of dadih lactic bacteria, *Lc. lactis* subsp. *lactis* IS-10285, IS-7386, IS-16183, IS-11857 and IS-29862, *L. brevis* IS-27560, IS-26958, *IS-* IS-23427, *Leu.mesen.mesenteroides* IS-27526, and *L. casei* IS-7257 each had a moderate survival rate (in a range of 4.83-5.49 log cfu/ml) for 2 h at pH 2.0, while commercial starter such as *L. casei* Rolly C, *S. thermophillus* Rolly T, *Bif. breve* Rolly B also had the same range of survival, which was 5.34, 5.41, 5.33 log cfu/ml, respectively (Table 1). Acid tolerant strains have an advantage for survival in stomach (pH 2.0 in extreme cases), to be able to resist the digestion process in the stomach, where hydrochloric and gastric juice are secreted.

Bile-salt tolerance of dadih lactic bacteria strains, *Lc. lactis* subsp. *lactis* IS-10285, *Lc. lactis* subsp. *lactis* IS-7386, *Lc. lactis* subsp. *lactis* IS-16183, and *Lc. lactis* subsp. *lactis* IS-29862 are bile-salt resistant, as indicated by a growth delay less than 10 min, recorded in the presence of 0.3% oxgall (w/v) (Toit et al., 1998), and the other six strains of dadih lactic bacteria were considered as bile-salt tolerant with a lag time value in a range of 5.03-11.82 min (Table 2). The three commercial strains, *L. casei* Rolly C, *S. thermophilus* Rolly T, *Bif. breve* Rolly B, each had a lag time of 22.2 min, 4.98 min and 12.36 min, respectively (Table 2). Bile-salt tolerance is important for strains to grow and survive in the upper small intestine, where BSH activity

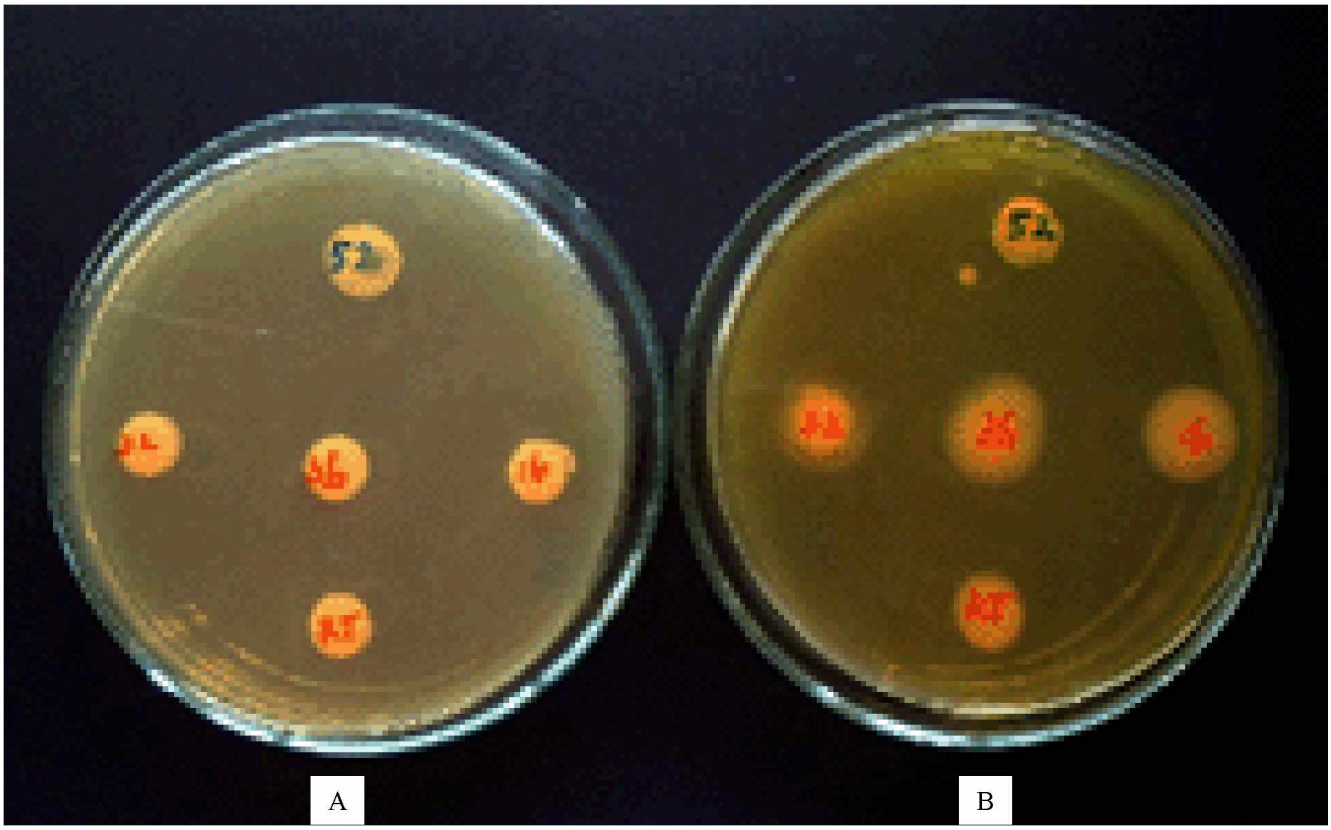

Figure 1. Bile salt hydrolytic activity as detected by the disc assay. A: MRS as control, and B: MRS supplemented with 0.5% TDCA and 0.37 g/l $CaCl_2$. (RT) *S. thermophilus* Rolly T, (16) *L. brevis* IS-26958, (52) *L. casei* IS-7257, (32) *Lc. lactis* subsp. *lactis* IS-11857, and (36) *Lc. lactis* subsp. *lactis* IS-29862

of such lactic bacteria may play a role in the enterohepatic cycle.

L. brevis IS-26958, Lc. lactis subsp. lactis IS-11857 and Lc. lactis subsp. lactis IS-29862 displayed the largest precipitation zones while screened for BSH activity (Table 2 and Figure 1). De Smet et al. (1994) suggested that lactobacilli with high BSH activity may contribute to a reduction of cholesterol levels, as the free bile salts (deconjugated) are not as easily reabsorbed in the intestines as conjugated bile salts, and may be excreted in the feces as poly-bile acid polymers (Benson et al., 1993). De Smet et al. (1995) also hypothesized that BSH activity may be important for bile-salt resistance, and may therefore be considered as an important colonization factor.

Among ten strains of dadih indigenous lactic bacteria, one strain, *Lc. lactis* subsp. *lactis* IS-16183 had good survival rate (8.11 log cfu/ml) in the presence of lysozyme after 60 min incubation, which is relatively stable as compare to the control (0 h) (Table 3). *Lc. lactis* subsp. *lactis* IS-7386, *Lc. lactis* subsp. *lactis* IS-11857, *Lc. lactis* subsp. *lactis* IS-29862, *Lactococcus lactis* subsp. *lactis* IS-16183, *Lactobacillus brevis* 23427 and *L. casei* IS-7257 had moderate to good survival rates in the range of 7.11-8.11 log cfu/ml, in the presence of lysozyme after 60 min

Table 3. Survival of dadih lactic isolates in the presence of lysozyme

LAB*	Lysozyme tolerance	
	Log cfu/ml	
	0 h	1 h
RC	8.11±0.11	6.96±0.19
RT	8.14±0.07	6.82±0.21
RB	8.1±0.13	6.74±0.11
IS-10285	8.27±0.22	6.64±0.14
IS-7386	8.22±0.19	7.48±0.18
IS-16183	8.14±0.26	8.11±0.09
IS-27560	8.13±0.16	6.84±0.05
IS-26958	8.15±0.09	6.7±0.18
IS-27526	8.3±0.12	4.37±0.11
IS-11857	7.85±0.13	7.74±0.31
IS-29862	7.97±0.09	7.11±0.13
IS-7257	8.13±0.14	7.33±0.27
IS-23427	7.97±0.21	7.31±0.16

* Refer to Table 1

Cells suspended in 0.06 *M* phosphate buffer (pH 6.8) supplemented with 1% NaCl containing 100 µg/ml lysozyme incubated at 37°C for 1 h and control (0 h). Values are the means with standard deviation of three experiments.

incubation. *Leu. mesen. mesenteroides* IS-27526 had poor survival rate. 4.37 log cfu/ml (Table 3). In addition. the lysozyme concentration used in this study was higher than the physiological intestinal concentration.

For orally applied probiotics the conditions in the orogastrointestinal tract are the major selection criteria for microbial strains, and lysozyme in the oral cavity may lyse gram positive bacterial cells. Tables 1. 2. and 3 show that *Lc. lactis* subsp. lactis IS-10285. IS 16183. IS-11857 and IS-29862. *L. brevis* IS-27560 and IS-26958. *Leu. mesen. mesenteroides* IS 27526. and *L. casei* IS-7257. each was tolerant to lysozyme, acid and bile, indicating that those strains may reach the intestine alive after ingestion.

Antagonistic activity of the viable cells of all eight isolates was observed against *E. coli* 3301. *Listeria* ATCC 19112. and *S. aureus* IFO 3060 (data not shown). Strains that exert antagonism against undesired intestinal bacteria may interact to stabilize or control the intestinal microflora. The CFNS of *Lactococcus lactis subsp. lactis* IS-10285, IS-16183, *L. brevis* IS-26958. and *L. casei* IS-7257 active against *E. coli* 3301 and *Listeria monocytogenes* ATCC 19112. and *L. brevis* IS 26958 also active against *S. aureus* IFO 3060 (Table 2). The assumed bacteriocin remained stable at pH 2-10. suggesting that it would be active at the pH of the GIT. Those strains which have no active CFNS against pathogenic bacteria might have different mechanisms in inhibiting the pathogenic bacteria, probably due to hydrogen peroxide and/or lactic acid produced.

CONCLUSIONS

All of the ten strains of indigenous dadih lactic bacteria originated from Bukit Tinggi - West Sumatra were acid and bile acid tolerant *in vitro*; hence. they are candidate probiotic strains which have antimutagenic and hypocholesterolemic properties. Further *in vivo* and human studies for their probiotic properties. such as hypocholesterolemic. antimutagenic and antipathogenic bacteria is underway. so that the successful candidate could be applied as starter culture of Indonesian fermented milk as functional drink.

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