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Taurocholate Deconjugation and Cholesterol Binding by Indigenous Dadih Lactic Acid Bacteria

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High serum cholesterol levels have been associated with an increased risk for human coronary heart disease. Lowering of serum cholesterol has been suggested to prevent the heart disease. To reduce serum cholesterol levels one may consumed diet supplementat of fermented dairy product such as dadih. Lactic acid bacteria present in dadih may alter serum cholesterol by directly bind to dietary cholesterol and/or deconjugation of bile salts. Acid and bile tolerance, deconjugation of sodium taurocholate, and the cholesterol-binding ability of lactic acid bacteria from dadih were examined. Among ten dadih lactic acid bacteria tested, six strains namely I-11, I-2775, K-5, I-6257, IS-7257, and B-4 could bind cholesterol and deconjugate sodium taurocholate. However, the last four strains were very sensitive to bile. Therefore, *Lactobacillus fermentum* I-11 and *Leuconostoc lactis* subsp. *lactis* I-2775 those were tolerant to acid and oxgall (bile) and deconjugated sodium taurocholate and bound cholesterol could be recommended as probiotic to prevent coronary heart disease.

INTRODUCTION

Several studies in the past decade have indicated that lactic acid bacteria (LAB) and their fermented products impart both nutritional and therapeutic benefits including hypocholesterolemic effect (Gilliland 1989; Rodas *et al*. 1996). Gilliland *et al*. (1985) proposed that LAB in fermented and nonfermented milk products were directly responsible for the hypocholesterolemic ability. They found that when *Lactobacillus acidophilus* was grown under proper conditions it could uptake the cholesterol from laboratory media. The conditions required for cholesterol uptake include anaerobic environment and the presence of bile. Those conditions were found in the intestinal tract (Gilliland 1989). Cholesterol binding by LAB in the small intestine may reduce the amount of dietary cholesterol absorbed. This proposed mechanism was widely accepted by many researchers (Hosono & Tono-oka 1995; Gopal *et al*. 1996; Usman & Hosono 1999b). To test the hypothesis, pigs were fed by cholesterol-enriched diet supplemented with a strain of *L. acidophilus* that actively bound cholesterol. The result showed that lower serum cholesterol occurred in *L. acidophilus* supplemented diet compared to the conlactobacilli supplement (Gilliland *et al*. 1985). This finding has been supported by the results reported by Danielson *et al*. (1989) showing that *L. acidophilus*, which was actively bound cholesterol from a laboratory medium, would exert a hypocholesterolemic effect in swine. Consumption of *Bifidobacterium longum*, which has similar abilities in laboratory test, reduced the concentration of high serum cholesterol (Rasic *et al*. 1992). There is a great variation among *L. acidophilus* strains and other LAB in the ability to bind cholesterol (Gilliland *et al*. 1985; Hosono & Tono-oka 1995; Usman & Hosono 1999a). This variation should be considered when cultures are being selected for probiotic or food.

Dadih, a traditional fermented milk product, is the most popular dairy product among people living in West Sumatera, Indonesia. LAB that involve in the fermentation of dadih are *Leuconostoc mesenteroides, Streptococcus faecalis, S. lactis* supsp. *lactis*, *S. cremoris*, *L. casei* subsp. *casei*, and *Lactobacillus casei* subsp. *rhamnosus* (Hosono *et al*. 1989). Antimutagenicity and binding activities of these LAB toward some heterocyclic amines, *N*-nitroso compounds and mutagenic heated foods have been studied intensively by Hosono and co-workers (Hosono *et al*. 1990; Surono & Hosono 1996). However, few reports exist on the cholesterol-binding activity of LAB isolated from dadih (Hosono & Tono-oka 1995). To our knowledge, no evidence is available on the bile salt-deconjugating ability and bile and acid tolerance of these LAB.

The present study was conducted to evaluate the ability of LAB isolated from dadih to bind cholesterol, to deconjugate sodium taurocholate and to assess bile and acid tolerance.

MATERIALS AND METHODS

Source and Maintenance of Cultures. The ten strains of LAB isolated from dadih used in this study were obtained from Research Center of the Indonesia Institute of Technology, Jakarta, Indonesia. All LAB were maintained by subculturing in de man rogosa and sharpe (MRS) broth using 1% inocula and 18 to 20 h of incubation at 37 °C; between transfer cultures were stored at 4 °C. Each culture was subcultured twice in MRS broth prior to experimental use.

Assay for Acid Tolerance. Washed cell pellets of each culture of LAB were resuspended in sterile distilled water and the absorbance (625 nm) was adjusted at 0.7. Each cell suspension at the level of 2% were inoculated into 10 ml of

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2% skim milk that had been adjusted to pH 3 with concentrated HCl and then incubated at 37 °C for 2 h. Immediately after incubation, 0.5 ml of suspended cells were diluted with 4.5 ml of phosphate buffered saline 66 mM, pH 6.8. Subsequent serial dilutions were made and plated by the spread-plate method with MRS agar. The plates were incubated at 37 °C for 48 h before enumeration. The experiments were repeated twice with duplicates each time. The means of data from two trials were presented.

Assay for Bile Tolerance. To study the effect of bile salts on the growth rate of LAB, a method described by Walker and Gilliland (1993) was employed. All cultures were evaluated for growth in MRS-THIO broth (MRS supplemented with 0.2% sodium thioglycolate) with and without 0.3% oxgall. Freshly prepared cultures were inoculated (1%) into each medium, incubated at 37 °C in a waterbath, and the growth was monitored hourly by using spectrometer at 620 nm. The growth was prolonged 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 reach the condition both in MRS-THIO broth with and without 0.3% oxgall. The difference in time (h) between the culture media was considered as the lag time (LT).

Analysis for Sodium Taurocholate-Deconjugating Activity. Deconjugation of sodium taurocholate was assayed in MRS-THIO broth (MRS plus 0.2% sodium thioglycholate) supplemented with 0.2% sodium taurocholate. The broth medium was inoculated with 1% of an active culture and incubated at 37 °C for 18 h. Analysis for the free cholic acid that was released during deconjugation was carried out according to the method of Walker and Gilliland (1993). Twenty-millimeter volumes of each strain were adjusted to pH 7.0 with 1 N NaOH. The volume was increased to 25 ml with distilled water and was centrifuged at 12,000 rpm for 10 min to remove the cells. Fifteen ml of the resulting supernatants were adjusted to pH 1.0 by using 10 N HCl and were further adjusted to 24 ml with distilled water. To a glass-stopper tube, three ml of these sample and 9 ml of 99.5% ethyl acetate were added and mixed thoroughly, and the phases were allowed to separate. Three ml of the ethyl acetate layer from each tube were transferred to a clean test tube containing 1 ml of 0.01 N NaOH, 6 ml of H_2SO_4, and 1 ml of 1% (w/v) fulfuraldehyde were added and mixed well. The tubes were heated at 65 °C for 13 min and then cooled to room temperature. Five ml of glacial acetic acid were added and then measured using a spectrophotometer. The concentration of free cholic acid in the supernatants was determined from a standard curve. Uninoculated medium was used as a control. All experiments were carried out with three replicates, and the results were expressed as micromoles of cholic acid per millimeter.

Assay for Cholesterol-Binding Activity. Sheep serum were added to MRS broth containing 2% sodium thioglycholate and 0.3% oxgall (MRSO broth) to obtain a concentration of cholesterol of 100 µg/ml. Five ml of MRSO broth were distributed into sterile tubes and then inoculated individually with 100 ml of active cultures. After incubation at 37 °C for 18 h, cells were removed by centrifugation at 2,000 rpm for 10 min. The spent broth was collected and analyzed for cholesterol with the method described by Rudel and Morris (1974). One tenth ml of the supernatant, 0.3 ml of (w/v) KOH, and 3 ml of 95% ethanol were placed in a capped tube, mixed thoroughly at 60 °C water bath for 15 min. After the mixture cooled to room temperature, 5 ml of hexane and 3 ml of distilled water were added, and the mixture was shaken for 1 min to ensure complete mixing. One ml of the hexane layer was pipetted into duplicate test tubes and the solvent was evaporated under nitrogen gas. Next, 2 ml of 0.05% (w/v) o-pthalaldehyde reagent were added to each tube and the solution was mixed until the sample was completely dissolved. After 10 min, 1 ml of concentrated H_2SO_4 was added; the solution were immediately mixed on a tube vibrator, and absorbance was measured with a spectrophotometer at 550 nm. The control solution was assayed using the same procedure but from the medium without inoculation. The cholesterol-binding ability was estimated by the following formula:

$$A = 100 - [(B/C) \times 100]$$

where A: binding of cholesterol (%), B: cholesterol (µg) in the supernatant of the inoculated MRSO broth, C: cholesterol (µg) in the supernatant of MRSO broth without inoculation (control).

RESULTS

Acid Tolerance. The number of colonies among the ten strains, showed a wide variation at pH 6.8, eventhough the initial absorbance of each cell suspension was relatively the same (Table 1). The highest number of colony was resulted in *L. lactis* subsp. *lactis* I-2775 (8.36 $\log_{10}$ cfu/ml), while the lowest one was *L. fermentum* I-11 (3.38 $\log_{10}$ cfu/ml). At pH 3.0, the viability of the ten strains decreased by 1 to 5 log cycles after 2 h incubation. *L. mesenteroides* I-6257 was the most sensitive strain to acid indicated by the decrease in number of viable cells by 5 log cycles. *L. plantarum* IS-7257 and *L. fermentum* I-11 were tolerant to acid because the number of viable cells decreased by only 1 log cycle.

Table 1. Acid tolerance of lactic acid bacteria isolated from dadih

Lactic acid bacteria	Number of colony after preincubated at different pH ($\log_{10}$ cfu/ml)	
	pH 6.8*	pH 3.0**
Lactococcus lactis subsp. *lactis* K-5	8.04	5.36
L. lactis subsp. *lactis* B-4	7.93	5.30
L. lactis subsp. *lactis* I-2775	8.36	6.34
L. lactis subsp. *lactis* IS-10285	8.30	6.34
Lactobacillus plantarum IS-7257	8.65	7.18
L. fermentum I-11	3.38	2.80
L. brevis IS-11857	8.23	6.28
L. plantarum IS-70386	6.42	4.87
L. casei I-5772	7.46	4.92
Leuconostoc mesenteroides I-6257	8.00	3.04

*Initial pH of 2% skim milk, **2% skim milk was adjusted to pH 3.0 using concentrated HCl

Bile Tolerance. Among the ten strains tested, only *L. fermentum* I-11 and *L. lactis* subsp. *lactis* I-2775 were tolerant to oxgall or bile (Table 2). *Lactobacillus plantarum* IS-7257 grew in MRS-THIO without oxgall but the absorbance did not increase by 0.3 units after 9 h in the same medium supplemented with oxgall. The absorbance of *L. mesenteroides* I-6257 grown in MRS-THIO plus oxgall decreased after 9 h of incubation indicating that this strain could not survive in bile containing media.

Deconjugation of Sodium Taurocholate. Among the ten strains, only six strains could deconjugate taurocholic acid by deliberating cholic acid (Table 3). The range activity was from 0.21 µmol/ml by *L. lactis* subsp. *lactis* I-2775 up to 0.45 µmol/ml by *L. lactis* subsp. *lactis* K-5. The other four strains namely IS-10285, IS-11857, IS-70386, and I-5772 were not able to deconjugate taurocholic acid.

Binding of Cholesterol. Table 4 shows the results obtained for the binding of cholesterol by LAB isolated from dadih. All cultures displayed the capability of binding cholesterol with variations in binding percentages. *Lactobacillus lactis* subsp. *lactis* B-4 exhibited the highest cholesterol-binding ability by removing 15% cholesterol from medium broth, while *L. lactis* subsp. *lactis* I-2775 showed the lowest activity (5%).

DISCUSSION

Elevated serum cholesterol in humans is generally a risk factor correlated with development of coronary heart disease. Modification of diets such as supplementation of diet with fermented dairy products or LAB-containing dairy products is a way that may be helpful in reducing serum cholesterol. LAB may alter serum cholesterol by two proposed mechanisms; directly binding of dietary cholesterol in the small intestine before cholesterol can be absorbed into the body (Gilliland *et al*. 1985; Hosono & Tono-oka 1995) and deconjugation of bile acids to produce free bile acids (Walker & Gilliland 1993; Gopal *et al*. 1996). The results from this study showed that all ten strains of LAB isolated from dadih were able to bind cholesterol, but the binding abilities varied among the strains tested. Hosono and Tono-oka (1995) reported that binding of cholesterol to the cells of LAB from various fermented milk products varied among the strains and species, and suggested that differences in binding abilities were due to the chemical and structural properties of their cell-wall peptidoglycans. In our previous studies we found the same results working on *L. gasseri* strains (Usman & Hosono 1999b).

Deconjugation of bile acids may help reduced serum cholesterol in humans because deconjugated bile acids are excreted more rapidly than the conjugated forms (Chikai *et al*. 1987). Sodium taurocholate is a major bile salt in human and

Table 3. Deconjugation of sodium taurocholate by lactic acid bacteria isolated from dadih

Lactic acid bacteria	Broth pH*	Cholic acid released** µmol/ml
Lactococcus lactis subsp. *lactis* K-5	5.39	0.45
L. lactis subsp. *lactis* B-4	5.34	0.30
L. lactis subsp. *lactis* I-2775	6.16	0.21
L. lactis subsp. *lactis* IS-10285	4.73	ND***
Lactobacillus plantarum IS-7257	5.29	0.43
L. fermentum I-11	5.24	0.32
L. brevis IS-11857	5.10	ND***
L. plantarum IS-70386	5.37	ND***
L. casei I-5772	5.43	ND***
Leuconostoc mesenteroides I-6257	5.25	0.37

*pH of medium after incubation at 37 °C for 18 h; **All cultures were grown in MRS-THIO broth (MRS broth supplemented with 0.2% sodium thioglycholate); ***Cholic acid released was not detected

Table 4. Binding of cholesterol by lactic acid bacteria isolated from dadih

Lactic acid bacteria	Broth pH*	Cholic acid released** µmol/ml
Lactococcus lactis subsp. *lactis* K-5	4.17	11.76
L. lactis subsp. *lactis* B-4	4.18	15.31
L. lactis subsp. *lactis* I-2775	4.22	5.39
L. lactis subsp. *lactis* IS-10285	4.24	8.07
Lactobacillus plantarum IS-7257	4.24	12.01
L. fermentum I-11	4.40	6.99
L. brevis IS-11857	5.18	10.48
L. plantarum IS-70386	5.01	11.88
L. casei I-5772	4.67	5.93
Leuconostoc mesenteroides I-6257	4.60	11.26

*pH of medium after incubation at 37 °C for 18 h; **All cultures were grown in MRS-THIO broth (MRS broth supplemented with 0.2% sodium thioglycholate)

Table 2. Bile tolerance of lactic acid bacteria isolated from dadih

Lactic acid bacteria	Time required (h) to increase A_{620} nm 0.3 units		LT* (h)
	MRS-THIO without oxgall	MRS-THIO with oxgall	
Lactococcus lactis subsp. *lactis* K-5	>9.0a	>9.0a	NM***
L. lactis subsp. *lactis* B-4	>9.0a	>9.0a	NM***
L. lactis subsp. *lactis* I-2775	3.74	4.45	0.71
L. lactis subsp. *lactis* IS-10285	>9.0a	>9.0a	NM***
Lactobacillus plantarum IS-7257	5.04	>9.0a	NM***
L. fermentum I-11	3.50	4.12	0.62
L. brevis IS-11857	>9.0a	>9.0a	NM***
L. plantarum IS-70386	>9.0a	>9.0a	NM***
L. casei I-5772	>9.0a	>9.0a	NM***
Leuconostoc mesenteroides I-6257	>9.0a	D**	NM***

*Difference in lag time (LT) required for the cultures to reach absorbance at 620 nm by 0.3 units in MRS-THIO broth supplemented with 0.3% oxgall or without oxgall; a: Absorbance not increased by 0.3 units within 9 h of incubation at 37 °C; **Absorbance decreased after 9 h of incubation at 37 °C; ***Absorbance not measured because time required for the cultures to reach absorbance at 620 nm by 0.3 units in MRS-THIO broth supplemented with 0.3% oxgall or without oxgall was more than 9 h

carnivores (Uchida *et al*. 1977); therefore, it was used to study the bile salt-decunjugating ability of dadih's LAB. It was found that among ten strains tested, only six strains could deconjugate sodium taurocholate. The result from the present study is contradictory from the previous findings by some researchers who reported that all LAB isolated from human and animal feces or small intestine had the ability to deconjugate sodium taurocholate, eventhough their bile salt-deconjugating activities varied among the species and strains (Gilliland & Walker 1989; Gopal *et al*. 1996; Usman & Hosono 1999a).

Previous studies reported the ability some LAB such as lactobacilli (Lundeen & Savage 1990; Usman & Hosono 1999a, 2000), and bifidobacterium (Grill *et al*. 1995) to deconjugate bile salt. The ability of *L. reuteri* to deconjugate bile salts in *in vitro* resulted in hypocholesterolemic effect in mice (Taranto *et al*. 1998). Similar findings were reported by Hashimoto *et al*. (1999) and Usman and Hosono (2000) who found an increased excretion of fecal bile acids in rats fed with cholesterol-enriched diet supplemented with viable cells of *L. casei* or *L. gasseri*. Thus, in a steady-state condition, deconjugation of bile acids can lead to reduction of the body serum cholesterol by increasing the formation of new bile acids. The new bile acids are needed to replace those that have been escaped from enterohepatic circulation (Reynier *et al*. 1981). Deconjugated bile acids are also excreted more rapidly than are conjugated bile acids. To replace the excreted bile acids, more bile acids would have to be synthetized from cholesterol and as a consequence, body cholesterol levels decreased (Driessen & de Boer 1989).

Another factor that should be considered in selecting *L. acidophilus* as a probiotic culture or as a food adjunct is acid and bile tolerances to be able to survive, grow, and perform therapeutic activity in the intestinal tract (Salminen & von Wright 1993). Gilliland *et al*. (1984) reported that a culture of *L. acidophilus* with high bile tolerance is significantly better than a strain with low bile tolerance to increase the number of facultative lactobacilli in the upper part of calve small intestine. As probiotic, LAB will be in contact with the stomach at pH ranging from 2.0 to 8.0 (Hood & Zottola 1988). Thus, probiotic cultures must be survive in an environment with gastric acid when viable cells go through the gastrointestinal tract. Survival at pH 3 for 2 h and growing in medium containing 1,000 ppm of bile acids are considered standards for acid- and bile-tolerance of probiotic cultures (Itoh 1992; Gohran 1994). The present study showed that strain K-5, B-4, 7257, and I-6257 exhibited higher cholesterol-binding and bile salt-deconjugating abilities, however, they were very sensitive to bile. Hence, they could not be used as probiotic. *Lactobacillus fermentum* I-11 and *L. lactis* subsp. *lactis* I-2775 displayed lower cholesterol-binding and bile salt-deconjugating abilities, however they were bile and acid tolerant. Thus, they may survive the high acidity in the stomach and high concentration of bile compounds in the intestine when consumed. Therefore, these two strains could be used as probiotic cultures.

The present study suggests that consumption of dadih made from *L. fermentum* I-11 and *L. lactis* subsp. *lactis* I-2775 will be useful in reducing human serum cholesterol level. To generalize this conclusion, *in vivo* animal model experiments are necessary.

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