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Isolation and Characterization of Lactic Acid Bacteria from Legume Soaking Water of Tempeh Productions

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Abstract

The aims of this study were to isolate lactic acid bacteria from legume soaking water and to examine their ability to grow and produce acid in jack bean milk. Lactic acid bacteria were isolated from legume soaking water in five tempeh productions in Special Province of Yogyakarta, Indonesia. The identification of LAB was carried out based on morphological, physiological, and biochemical characteristics. Twenty-nine LAB were obtained, and twelve isolates considered to be homo-fermentative types. Four of these homofermentative isolates which be able to grow at pH 4.4 were further examined of their ability to grow and produce acid in jack bean milk. Biochemical identification using API 50 CH and 50 CHL identified them as *Lactobacillus* sp. KKNB1, *L. plantarum* WGK3, *L. plantarum* WGK4, and *L. paracasei* WGK5. All those isolates were able to ferment jack bean milk which was marked by an increase of the number of cells (1.07-1.76 log cycle) and decrease of pH value in jack bean milk after fermentation at 37°C for 24 hours. It means that they could utilize carbon sources and other nutrients in jack bean milk for their growth and metabolic activities. Further study should be done to evaluate the possibility of these isolates for starter cultures in fermentation of jack bean milk.

Keywords

legume soaking water, lactic acid bacteria, isolation, identification, jack bean milk fermentation

1 Introduction

Lactic acid bacteria play important roles in various non-dairy fermented foods in Asian countries such as *kimchi*, *nham*, and *balao-balao* [1]. Most of Indonesian traditional fermented foods are the product of spontaneous fermentation, involving lactic acid bacteria that occur naturally in raw materials and environment such *urutan* and *petis daging* (fermented meat), *bekasam* (fermented fish), and *ronto* (fermented shrimp) [2, 3]. There are various roles of lactic acid bacteria in fermented foods such as to produce better flavor, aroma, and texture, improving the nutritional values, safety, and some functional compounds. Their ability in lowering pH due to the production of lactic acid proves to have beneficial effect to the fermented products such as more stable product with longer shelf live.

The involvement of lactic acid bacteria in foods could be grouped into three different aspects: as a starter culture for fermentation of food, as bio-preservatives, and as a component for probiotic food. A combination of these three aspects is of course possible, such as using probiotic strains that also have antimicrobial agents or probiotic as a starter culture. The addition of proteolytic *L. plantarum* S4512 as a starter culture in sorghum flour fermentation suppressed the growth of coliforms and increased significantly the proteolytic activity and therefore the soluble protein [4]. The addition of *L. plantarum* B 1765 isolated from spontaneous *bekasam* fermentation as a starter culture increased the ACE inhibitor activity of the *bekasam* compared to the original *bekasam* [5]. Lactic acid bacteria isolated from fermented food such as tempeh, and *bakasang*, and from buffalo milk showed some antimicrobial activity against pathogenic bacteria [6,7,8].

There are many studies reported that lactic acid can grow not only in cow-milk but also in legume milk such as soymilk and peanut milk [9,10]. Lee and Beuchat [11] reported that fermentation of peanut milk with lactic acid bacteria removes hexanal, one of the compounds responsible for the unwanted beany flavor in peanut milk. Some studies showed that strains of lactic acid bacteria improved antioxidant properties of

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fermented soymilk, *kerandang* milk (*Canavalia virosa*) and sesame milk [12,13]. Nocianitri et al. [14] isolated lactic acid bacteria from healthy infant fecal material and found 12 isolates that could grow in soymilk.

The ability of lactic acid bacteria to grow in legumes milk indicates that they can utilize available carbohydrates in legume milk. Indonesia has various under-utilized legumes that are cooked and consumed locally. One of them is jack bean (*Canavalia ensiformis* (L.) DC.). Like soybean, jack bean can be processed into jack bean milk and fermented by suitable lactic acid bacteria to produce fermented jack bean milk. In milk fermentation, the main fermentable sugar is lactose, thus lactic acid bacteria that produce β-galactosidase can hydrolyze lactose into fermentable sugars of glucose and galactose. On the other hand, beside sucrose, legumes contain oligosaccharides such as stachyose, raffinose and verbascose that should be degraded by α-glucosidase before being used by lactic acid bacteria. Pisol et al. [9] isolated various types of lactic acid bacteria from every stage of tempeh production. Usually, tempeh is made from soybean, but in some areas in Indonesia, tempeh also can be made from other legumes such as white lima bean, red lima bean and velvet bean. It could be that those various legumes soaking water contain lactic acid bacteria. Thus, the aim of this study was to isolate and identify lactic acid bacteria from legume soaking water in various traditional tempeh productions and to examine their ability to grow and produce acid in jack bean milk.

2 Materials and Methods

2.1 Sample collection

Five samples of legume soaking water were collected from five different local tempeh productions in the Special Province of Yogyakarta (Daerah Istimewa Yogyakarta), Indonesia as seen in **Table 1**. Legume soaking water samples were taken aseptically and put in a sterile glass bottle and brought to the laboratory using thermal insulated bags with ice gel. Each sample was measured for its pH and the amount of LAB were enumerated immediately after its arrival at the laboratory.

Table 1 Sample sources for LAB isolation

Code	Location of tempeh production	Legume soaking water
KKN	Gentan, Sleman, DIY	Soybean (*Glycine max* (L.))
KKP	Kalibawang, Kulon Progo, DIY	White lima bean (*Phaseolus lunatus*)
GKM	Gentan, Sleman, DIY	Red lima bean (*Phaseolus lunatus*)
WGK	Wintaos. Gunung Kidul, DIY	Red lima bean (*Phaseolus lunatus*)
WGB	Wintaos, Gunung Kidul, DIY	Velvet bean (Mucuna pruriens)

2.2 Isolation of LAB

Each 10 mL sample was aseptically added into 90 mL 0.85% sodium chloride solution. A series of dilutions using 0.85% sodium chloride solution was carried out. One milliliter of the last three dilution series of each sample was inoculated into de Man, Rogosa, Sharpe (MRS) agar (Oxoid Ltd) with the addition of 0.5% calcium carbonate ($CaCO_3$) and 0.5% L-cysteine hydrochloride using the pour-plate method. After MRS agar was solidified, Petri-dishes were put into a double lock zipper plastic bag in an upside-down position. The air in the plastic bag was removed using a deflator electric air pump, then nitrogen gas was filled in. Incubation was carried out at 37°C for 48 hours. Bacterial colonies that formed a clear zone were picked-up and purified using a series of dilutions and pour plate techniques with the same incubation method.

Single colonies obtained were purified using the same method. The purity of isolates can be observed by looking at the uniformity of the colonies' form and bacterial cell shape on the microscope. Each pure isolate was inoculated into MRS broth and incubated at 37°C for 48 hours. The cells were harvested, washed and added with the mixture of sterile 20% sucrose and 10% skim milk with a ratio of 1:1 (v/v), and then stored in a freezer at -20°C.

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2.3 Identification of LAB

The identification of LAB was carried out based on morphology (cell shape, Gram staining, and motility) and biochemical characteristics (catalase and gas production from glucose) [15]. The gram-positive cell was indicated with blue-purple color meanwhile gram-negative cells with red color. Catalase test was performed by adding freshly prepared hydrogen peroxide (H_2O_2) 3% (v/v) onto cultured colonies. The formation of the bubble indicates the catalase-positive of the isolate. A motility test was carried out by inoculation of each culture onto semi-solid media using inoculating needle and incubation at 37 ℃ for 18-24 hours. If the growth of bacteria just in the point of inoculation, they are considered as non-motile bacteria, but if the colony spread out of the point of inoculation then they are motile ones. The gas production test was carried out by inoculation of 100 µl bacterial culture into 5 mL MRS broth in a test tube with an upside-down Durham tube inside. Incubation was carried out at 37°C for 24-48 hours and gas production was monitored.

The physiological test was conducted by examining the ability of the isolates to grow in MRS broth at various temperatures (10°C, 20°C and 42°C), pH (4.4 and 9.6) and NaCl with the concentrations of 6.5% and 18%. Observations were done by measuring the absorbance of bacterial cultures at 660 nm at initial and after incubation for 24 and 48 hours on the test media using a spectrophotometer (Halo SB-10 Dynamic Scientific Ltd). The ability of bacterial growth is characterized by an increase in the absorbance of bacterial cultures to more than 1.00 at 24 and 48 hours, which then marked as (+).

Selected homo-fermentative LAB that could grow in MRS broth with pH 4.4 then further identified using API 50 CH and 50 CHL kits (Bio-Merieux, France) based on the method on manufacture's instruction. Data were obtained after inoculation of isolates in the kit and cover it with sterile paraffin, followed by incubation at 37°C for 48 hours. Color changes from dark blue-purple to yellow or greenish indicated sugar fermentation activity of isolates tested, then was marked (+), no color changes were marked (-), and if discoloration but not firm then was signed (?). Data then were put in API Web database system for interpreting the results.

2.4 Test for the growth and acid production of selected LAB in jack bean milk

Four selected homo-fermentative LAB that can grow in pH 4.4 were examined for their ability to grow and produce acid in jack bean milk. Jack bean milk was extracted from jack bean seed with cooked peeled jack bean and water ratio of 1:4 (w/v) [16]. Pasteurized jack bean milk was inoculated with 1% (v/v) each of 18 hours selected LAB and incubated at 37°C for 24 hours. In the initial and end of fermentation time, the number of viable cells was enumerated, and the pH was measured. The pH of the legume soaking water and pH of fermentation media were measured using pH meter (Hanna HI 2210). The population of lactic acid bacteria was enumerated using dilution and pour plate method on MRS agar with 0.5% $CaCO_3$ and incubation at 37° C for 48 hours. Colonies with a clear zone that appeared on the plates were counted and expressed as colony-forming units (CFU/mL).

3 Results and Discussion

3.1 Isolation and purification of lactic acid bacteria

Lactic acid bacteria were isolated from soaking water in Tempeh production using raw materials of soybean, white lima bean, red lima bean and velvet bean from five different places with the code of KKN, KKP, GKM, WGK, and WGB respectively as illustrated in **Table 2.** The pH of the soaking water sample ranges from 4.6-5.89. The lowest pH value was found in soybean soaking water of 4.6 and the highest one obtained from velvet bean soaking water.

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Table 2 pH and number of LAB in legume soaking water

Code	pH	Number of LAB (CFU/mL)
KKN	4.60	6.3×10^8
KKP	4.81	2.9×10^7
GKM	5.10	1.8×10^7
WGK	4.80	3.5×10^6
WGB	5.89	2.2×10^6

The number of lactic acid bacteria in the soaking water ranges from 2.2×10^6 CFU/mL in velvet bean soaking water to 6.2×10^8 CFU/mL in soybean soaking water. The result shows that there is a correlation between the number of LAB and the pH value. The highest number of LAB and the lowest pH value was found in soybean soaking water, meanwhile, the lowest number of LAB and the highest pH value was obtained in velvet bean soaking water, similar to the result reported by Pisol et al. [17] with the number of LAB in the day 1 soybean soaking water in the range of 7.35-7.56 log CFU/mL. Meanwhile, Amaliyah et al. [18] found that the number of cells in soybean soaking water in the process of making tempeh was 9.17×10^8 CFU/mL. The presence of lactic acid bacteria in the soaking water is very important because the growth and metabolic activity of lactic acid bacteria produce acid and lower the pH of the soaking water. This acid condition can inhibit the growth of pathogenic bacteria.

3.2 Identification of lactic acid bacteria

Twenty-nine isolates were considered as Gram-positive, non-motile, and catalase-negative with 26 isolates were rod shapes and only 3 isolates were cocci. Based on the ability to produce gas from glucose, 17 isolates and 12 isolates were classified as hetero-fermentative and homo-fermentative lactic acid bacteria respectively **Table 3**. Pisol et al. [19] isolated 16 LAB from different stages of tempeh production and found Lactobacillus heterofermentative was dominant in every stage of tempeh production.

Table 3 LAB morphological and biochemical test results

Source	Number of isolates						
	Gram stain	Motility	Catalase	Morphology		Gas Production	
				Rod	Cocci	Positive	Negative
KKN	11	11	11	11	-	10	1
KKP	5	5	5	2	3	1	4
GKM	5	5	5	5	-	3	2
WGK	4	4	4	4	-	1	3
WGB	4	4	4	4	-	2	2
Total	29	29	29	26	3	17	12

Homo-fermentative LAB is more suitable as a starter culture for fermentation of dairy and non-dairy milk because they produce mainly lactic acid bacteria and no gas production. Therefore, homo-fermentative LAB was examined their physiological properties such as their ability to grow at various temperatures, pH and salinity. The physiological properties of homo-fermentative LAB can be seen in **Table 4** All of the LAB isolates could not grow in 10°C and pH of 9.6. Only lactic acid bacteria from the genus of *Aerococcus*, *Enterococcus*, and *Tetrageonococcus* can grow in pH 9.6 [20]. Since LAB isolates are going to be used as a starter culture for fermentation of jack bean milk, therefore LAB that can grow at pH 4.4 was selected for further study. Four isolates namely KKNB1, WGK3, WGK4, and WGK5 could grow at pH 4.4. The four isolates were rod-shaped. KKNB1 isolate was unable to grow at a temperature of 20°C and grow well at 45°C and did not grow in 6.5% salinity. The other three isolates could grow at 20-45°C and 6.5% salinity.

Lactic acid bacteria isolated from Indonesian fermented foods are dominated by *Lactobacillus plantarum*, followed by *Pediococcus pentosaceus*, and *Streptococcus thermophilus*. *Lactobacillus* species exist in most of fermented food studies. *Pediococcus* species exist in several foods, while *Streptococcus, Enterococcus* and *Leuconostoc* species were mostly isolated from salted fermented fish and shrimp [21]. Lactic acid bacteria with proteolytic activity have also been isolated from spontaneously fermented soymilk [22], and spontaneously fermented sorghum flour [23], and they are and considered to be strains of *L. plantarum*. Wikandari et al., [24] isolated 150 lactic acid bacteria strains from *bekasam*, and more than half of them have proteolytic activities which belong to species of *L. plantarum*, *L. pentosus*, and *Pediococcus pentosaceus*.

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Table 4 Phenotypic characteristic of homo-fermentative LAB

Isolat code	Morphology	Temperature			NaCl		pH		Genus (tentative)
		10°C	20°C	45°C	6.5%	18%	4.4	9.6	
KKNB1	Rod	-	-	+	-	-	+	-	*Lactobacillus*
KKP1	Rod	-	-	+	-	-	-	-	*Lactobacillus*
KKP3	Cocci	-	+	+	-	-	-	-	*Streptococcus*
KKP4	Cocci	-	+	+	-	-	-	-	*Streptococcus*
KKP5	Cocci	-	+	+	-	-	-	-	*Streptococcus*
GKMB2	Rod	-	-	+	+	-	-	-	*Lactobacillus*
GKMB4	Rod	-	-	+	-	-	-	-	*Lactobacillus*
WGK3	Rod	-	+	+	+	-	+	-	*Lactobacillus*
WGK4	Rod	-	+	+	+	-	+	-	*Lactobacillus*
WGK5	Rod	-	+	+	+	-	+	-	*Lactobacillus*
WGB2	Rod	-	+	-	-	-	-	-	*Lactobacillus*
WGB4	Rod	-	+	-	-	-	-	-	*Lactobacillus*

From API-Kit identification test, it was found that the KKNB1 isolate had a similarity of 31.3% *Lactobacillus delbrueckii* ssp lactis 1 (**Table 5**). The low similarity was caused by KKNB1 isolates not being able to acidify lactose test media. Thus, KKNB1 isolates were stated as *Lactobacillus* sp. KKNB1 because according to characterization result, this isolate belongs to *Lactobacillus* genus. WGK3 and WGK4 isolates have a similarity of 99.9% with *Lactobacillus plantarum* 1, meanwhile WGK5 isolates have a similarity of 97.2% with *Lactobacillus paracasei* ssp paracasei 3. The results of this identification are the initial process in determining the type of bacteria at the species level. Therefore, further testings at the molecular level were needed for more accurate results.

Table 5 Identification of selected lactic acid bacteria isolated from legume soaking water using API 50 Ch and 50 CHL

Isolate	Identification result	Degree of accuracy
KKNB1	*Lactobacillus delbruckii* ssp lactis 1	31.1% identification not valid
	Weissella viridescens	24.7%
	Lactobacillus delbrueckii ssp delbrueckii	23.4%
	Pediococcus damnosus 1	10.4%
	Lactobacillus acidophilus 3	8.6%
WGK3	*Lactobacillus plantarum* 1	99.9% excellent identification
WGK4	*Lactobacillus plantarum* 1	99.9% excellent identification
WGK5	*Lactobacillus paracasei* ssp paracasei 3	97.2% doubtful profile

Based on API 50 CH and 50 CHL kits test based on API-Kit, all selected isolates could ferment glucose, fructose, and sucrose which are sugars found in jack bean. Among the other isolates, WGK 3 and WGK 4 isolates have the advantage of being able to use raffinose. These two isolates may have α-galactosidase activity. Raffinose is a type of oligosaccharide contained in jack bean and other legumes. Raw and cooked raffinose content in jack bean was 1.51 and 0.50 gram/ 100 gram respectively [25].

3.3 The ability of selected LAB to grow and produce acid in jack bean milk

All isolates were able to grow on jack bean milk at 37˚C as demonstrated in **Table 6**. The number of viable cells of *Lactobacillus* sp. KKNB1 increased about 1.76 log cycles after 24 hours incubation, whereas, the others increased about 1.07 to 1.30 log cycles. All selected isolates were also able to produce acid indicated by the reduction of pH to 5.02-5.20 during jack bean milk fermentation from the initial pH of 6.50. It could be that those isolates could utilize carbon sources and other nutrients in jack bean milk for their growth. There are some sugars in jack bean seed such as glucose, fructose, sucrose, and oligosaccharides including raffinose, stachyose, and verbascose [25]. Based on the API 50 CH test, only *L. plantarum* WGK3 and *L. plantarum* WGK4 could utilize raffinose. It could be that the other two isolates that could not utilize raffinose, consumed available sucrose in jack bean milk. Additionally, simple sugars in jack bean milk might

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improve the growth of lactic acid bacteria and the production of acid. Further study is needed to improve the performance of lactic acid bacteria in the fermentation of jack bean milk.

Table 6 Number of LAB, acid production and pH in jack bean milk after incubation at 37°C for 24 hours using selected LAB isolated from legume soaking water

Isolate	Cell growth (log cycle CFU/mL)	Initial pH	pH (24 hours)
Lactobacillus sp. KKNB1	1.76 ± 0.6	6,70 ± 0.00	5.05 ± 0.02
L. plantarum WGK3	1.21 ± 0.23	6.66 ± 0.01	5.20 ± 0.06
L. plantarum WGK4	1.07 ± 0.35	6.80 ± 0.01	5.10 ± 0.03
L. paracasei WGK5	1.30 ± 0.07	6.52 ± 0.01	5.02 ± 0.03

4 Conclusions

Twenty-nine lactic acid bacteria have been successfully isolated from legume soaking water in various tempeh production. Based on morphological characteristics, 26 isolates were bacilli and the rest were cocci shapes. Twelve lactic acid bacteria considered to be homo-fermentative types, indicated by no gas production. Four homo-fermentative lactic acid bacteria that could grow at pH 4.4 could grow in jack bean milk. Two isolates were identified as *L. plantarum*, and the others as *Lactobacillus* sp. and *L. paracasei*. They can grow in jack bean milk and produce acid as indicated by the decrease in pH after incubation at 37°C for 24 hours. Further study is needed to evaluate their ability as a starter culture for jack bean milk fermentation.

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