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Growth of indigenous lactic acid bacteria *Lactobacillus plantarum-pentosus* T14 and *Lactobacillus plantarum-pentosus* T35 in *kerandang* (*Canavalia virosa*) milk and changes of raffinose

Titiek Farianti Djaafar[1*], Muhamad Nur Cahyanto[2], Umar Santoso[2] and Endang Sutriswati Rahayu[2]

[1]The Assessment Institute for Agricultural Technology, Yogyakarta 55584, Indonesia.
[2]Faculty of Agricultural Technology, Gadjah Mada University, Yogyakarta 55281, Indonesia.
Email: titiekfd@yahoo.co.id

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ABSTRACT

Aims: *Kerandang* (*Canavalia virosa*) beans are good source of protein, yet predominantly *kerandang* foods are not widely accepted mainly because of their beany flavour the belief that they cause flatulence. The objectives of this research were to evaluate of viability of lactic acid bacteria from Indonesia indigenous fermented food in *kerandang* milk and its ability to metabolize indigestible oligossacharide raffinose.
Methodology and results: Two strains of Indonesia indigenous lactic acid bacteria (LAB), namely *Lactobacillus plantarum-pentosus* T14 and *Lactobacillus plantarum-pentosus* T35 were used for fermentation of *kerandang* milk. The results showed that all strains of lactic acid bacteria possess the ability to grow and produce of lactic acid in *kerandang* milk, indicated that total acid (TA) increase, pH decrease and their counts of LAB increase during fermentation period (0-24 h). The two strains of lactic acid bacteria were also able to metabolize raffinose into simple sugar (sucrose, glucose, fructose and galactose) during fermentation at 37 °C, however the raffinose transformation by *L. plantarum-pentosus* T14 more ability than *L. plantarum-pentosus* T35. The metabolism of raffinose during fermentation by *L. plantarum-pentosus* T14 and *L. plantarum-pentosus* T35 were 98.23% and 48.98%, respectively.
Conclusion, significance and impact of study: *Kerandang* milk fermented by lactic acid bacteria can decrease of saccharide raffinose cause of flatulence. Thus, lactic fermented of *kerandang* milk be safer for consumption.

Keywords: kerandang (*Canavalia virosa*), indigenous lactic acid bacteria, *kerandang* milk fermentation

INTRODUCTION

Kerandang (*Canavalia virosa*) belongs to the Family of the Fabaceae, Genus Canavalia and Species *Canavalia virosa*. *Kerandang* is a tropical crop, creeping, trilobed leaves with pink to purple flowers and fragrant. Flowers width is 3 cm, pods size 17 cm x 3 cm, old grain brown or reddish brown with black marble (Figure 1). The *kerandang* seeds contains protein 31.3%, fat 4.9%, 3.8% ash, and calory 1512.4 kJ/100 g (db), contains essential amino acids such as leucine, isoleucine, histidine, and methionine cysteine threonine and contains calcium, zinc, manganese and iron (Mukhopadhyay *et al.*, 1985; Shridar and Seena, 2005). *Kerandang* beans are good source of protein, yet predominantly *kerandang* foods are not widely accepted mainly because of their beany flavor the belief that they can cause flatulence. Numerous process, such as soaking, germination, hydrothermal processing and fermentation of *kerandang* can be lessen undesirable flavors during processing (Trugo *et al*., 1993; Sridhar and Seena, 2005; Djaafar *et al.*, 2010).

Beany flavors and unindigestible oligosaccharides component such as raffinose, stachyose and verbascose are obstacles to more widespread use of beans that may cause a gastrointestinal discomfort to consumers (Scalabrini *et al*., 1998; Shin *et al*., 2000). Raffinose is carbohydrate reserves in many plant tissues and particularly in seed. It is include one galactose unit, linked together with sucrose through α-1,6 linkages. Since humans are deficient in pancreatic α-galactosidase, raffinose are not digested in the duodenum. Therefore, the raffinose passes into the large intestine where they are degraded by gas-producing intestinal bacteria, such as *Clostridium* spp. and *Bacteroides* spp., yielding considerable amount of CH_2, CO_2 and H_2. The abnormal accumulation of flatulent rectal gas thus provokes gastrointestinal distress, such as abdominal pain, nausea, diarrhea and increased peristalsis (Silvestroni *et al.*, 2002).

***Corresponding author**

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Kerandang plants

Kerandang seeds

Peeled *Kerandang* seeds

Figure 1: *Kerandang* plants and seeds.

Lactic acid bacteria (LAB) are found in a large variety of nutrient rich environments, including milk and dairy products, vegetable and plants, cereals, meat and meat products. Many species are used for the manufacture and preservation of fermented foods from raw agricultural materials in which they are usually added as starters in order to control the fermentations. Lactic acid bacteria are producing of α-galactosidase, as catalyst the hydrolysis of oligosaccharide into monosaccharide that enters the glycolytic pathway, such as conversion of raffinose into sucrose and galactose. Glucose and fructose form sucrose by the action of sucrase and then into the glycolytic pathway. Whereas, galactose can be converted to glucose 1-phosphate in the Leloir pathway and then enter the glycolytic pathway (Atlas, 1997). The ability of lactic acid bacteria to ferment the available carbohydrate in a growing medium varies with strains.

The utilization of lactic acid bacteria in preparing soymilk fermentation has received much attention (Tsangalis *et al.*, 2002; Tsangalis and Shah, 2004; Chun *et al.*, 2007). Several study on metabolism of α-galactosyl oligosaccharides by *Lactobacillus* and *Bifidobacterium* strains in soymilk have been reported but in *kerandang* milk has not been done yet. The objectives of this research were to evaluate the growth of lactic acid bacteria in *kerandang* milk and investigate its ability to hydrolyze of indigestible oligossacharide raffinose into sucrose and galactose.

MATERIALS AND METHODS

Kerandang beans

Kerandang beans were obtained from the wild plants growing in sand beach land in the Bugel Village, Panjatan District, Kulon Progo Regency, Yogyakarta. Harvesting was conducted by picking old brown pods from the trees. Pods were peeled. Peeled beans were dried until 10% water content and their epidermis seeds were further removed mechanically by using an abrasive peeler to produce yellowish clean peeled beans.

The saccharides in *kerandang* beans were analyzed using HPLC (Vega *et al*., 2009). *Kerandang* bean flour (1 g) was homogenized in aqueous ethanol (25 mL, 80%, v/v), vortex for 1 min and placed in waterbath at 50 ºC for 30 min. The ethanol extracts were recovered, concentrated under vacuum, and the water phase was frozen and lyophilized. The saccharide were redissolved in deionized water, the prepared samples were purified by passage through a millex 0.45 µM. The concentrations of raffinose, sucrose, galactose, glucose and fructose were determined with a Varian HPLC fitted with a Metacarb 87C column and corresponding guard column maintained at 85 °C and an RI detector (1260 RID). The eluent is H_2O and flow rate of 0.6 mL/min. A 40 µL injection volume was used for both samples and standards. The retention times of the standards for raffinose, stachyose sucrose, galactose, glucose and fructose (Sigma) were at 6.88; 6.42; 7.65; 10.68; 9.52; 11.99, respectively. Standard stock solutions of raffinose (10 000 ppm), sucrose (10 000 ppm), galactose (10 000 ppm), glucose (1 000 ppm) and fructose (10 000 ppm) were used for preparation of standard calibration curve. The concentration of saccharide were derived from the standard curve and was expressed as part per million (ppm).

Cultures

Two strains of lactic acid bacteria were isolated from tempeh and bamboo shoot pickle. *L. plantarum-pentosus* T14 and *L. plantarum-pentosus* T35 were obtained from FNCC (Food Nutrition Culture Collection) Gadjah Mada University, Yogyakarta. The culture stock were kept in 10% glycerol and 10% skim milk with the ratio 1:1. One mililiter culture in sterile 1.5 mL polyethylene screw cap tube was added with 1 mL glycerol-skim milk and stored at –40 °C. The strains were rejuvenated in MRS (De Mann Rogosa Sharpe) broth (Oxoid) at 37 °C for 24 h.

Preparation of *Kerandang* milk

Preparation of *Kerandang* milk was conducted as described by Chun *et al.* (2007). Whole beans were washed and soaked for 3 h in water. The ratio of dry beans to water was 1:6 (w/v). The soak water was decanted and the beans were washed. The swollen beans were ground with hot water (90 °C) by using a Waring blender for 2 min at high speed. The ratio of dry

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beans to water used for grinding was 1:10 (w/v). The slurry was filtered through a double-layer cheese cloth. The resultant *Kerandang* milk was dispensed in 100 mL screw cap bottles and then pasteurized for 20 min at 80 °C.

Fermentation of *Kerandang* milk

The test inoculums was prepared by transferring the cultures from MRS Broth medium into *Kerandang* milk and subcultured in the same medium twice, incubation at 37 °C for 20-24 h. One hundred and fifty milliliters (150 mL) of *kerandang* milk were inoculated with single culture (0.2%, v/v) and then incubation at 37 °C for 24 h. Each bottle were taken out and sampling aseptically at interval 6 h during fermentation. Sample were directly analyzed for pH using pH meter, total acid with titrable acidity method and cell growth using plate count method on MRS agar (Fardiaz, 1992).

Determination of pH

The pH of the aliquots withdrawn every 6 h during the fermentation was monitored using a microprocessor pH meter (Thermo Scientific, Orion 3 Start) at 27 °C after calibrating with fresh pH 4.0 and 7.0 standard buffers.

Determination of total acid and cell growth

Titratable acidity was determined by the method of Fardiaz (1992) by titration with a 0.1 N NaOH solution an expressed as percent lactic acid. Cell numbers were measured in triplicates by using pour plate method (Fardiaz, 1992) with lactobacilli MRS media (Oxoid). Fermented samples (1 mL) were serially diluted with 0.85% NaCl solution and then 100 µL of diluted samples were taken into sterile plate. MRS medium with 1.5% agar and 0.8% $CaCO_3$ was poured into the plate and mixed carefully. After incubation at 37 °C for 24 h, single colonies were counted.

HPLC of saccharides

Estimate of raffinose and other saccharides were performed as previous methods of Vega *et al.* (2009). A 20 mL fermented *Kerandang* milk was centrifuged at 4,500 rpm for 15 min, 4 °C. The supernatant was stored at -20 °C until use for HPLC analysis.

One mL supernatant is put into the 5 mL flask and brought to volume with H_2O. The prepared samples were purified by passage through a millex 0.45 µM. The concentrations of raffinose, sucrose, galactose, glucose and fructose were determined with a Varian HPLC fitted with Metacarb 87C column and corresponding guard column maintained at 80 °C and an RI detector (1260 RID). The eluent is H_2O and flow rate of 0.6 mL/min. A 30 µL injection volume was used for both samples and standards. The retention times of the standards for raffinose, sucrose, galactose, glucose and fructose (Sigma) were at 6.88; 7.65; 10.68; 9.52; 11.99, respectively. Standard stock solutions of raffinose (10 000 ppm), sucrose (10 000 ppm), galactose (10 000 ppm), glucose (1000 ppm) and fructose (10 000 ppm) were used for preparation of standard calibration curve. The concentrations of saccharide were derived from the standard curve and was expressed as part per million (ppm).

RESULTS AND DISCUSSION

Saccharide in *kerandang* beans

Saccharides content in *kerandang* beans were presented in Table 1. The highest saccharide in *kerandang* beans is raffinose in the amount of 17334 ppm followed by sucrose 8311 ppm; stachyose 1684 ppm and fructose 1232 ppm. While, galactose and glucose are not detected in *kerandang* beans. All legumes, however, contain several oligosaccharides, among which raffinose, stachyose and verbascose are important (Abdel-Gawad, 1993; Trugo *et al.*, 1993). Onigbinde and Akinyele (1983) suggest that local variety of cowpea content 48 400 ppm stachyose and 41 200 ppm raffinose. According to Doss *et al.* (2011), *Canavalia ensiformis* contains 15100 ppm raffinose and 18000 ppm stachyose.

These oligosaccharides are generally undesirable due to flatulence effect. Human alimentary tract is deprived of α-galactosidase capable of hydrolyzing the α-1-6 galactoside linkage; therefore, these oligosaccharides are not digested and accumulate in large intestine where they undergone anaerobic fermentation by bacteria such as lactic acid bacteria. Thus, some gases are produced owing to fermentation, such as carbon dioxide, hydrogen and methane (Fleming, 1981; Alfred *et al*., 1982; Silvestroni *et al*., 2002).

Table 1: Saccharides of *kerandang* beans (ppm) as compared to soybean and *Phaseolus vulgaris* L var. Pinto Durango

Saccharides	*Kerandang*	Soybean[a]	*Phaseolus vulgaris* L var. Pinto Durango[b]
Raffinose	17,334	16,000	17,000
Stachyose	1,684	34,300	45,000
Sucrose	8,311	51,100	na
Glucose	nd	nd	na
Fructose	1,232	4,800	na
Galactose	nd	na	na

Nd: not detected; na: not analysis
[a] Concerton *et al.* (1983)
[b] Vega *et al.* (2009)

Growth of lactic acid bacteria in *kerandang* milk

Changes in viable cell number of the two strains of lactic acid bacteria in *Kerandang* milk during fermentation at 37 °C shown in Figure 2. In general, *L. plantarum-pentosus* T14 and *L. plantarum-pentosus* T35 showed a relatively

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good growth in *kerandang* milk. The initial cell growth rate varied slightly depending on the species. Initial cell population of 10^6 - 10^7 CFU/mL rapidly increased and reached 10^9 - 10^{10} CFU/ml in all *kerandang* milk after 6 to 12 h of fermentation and reached constant at 24 h fermentation. *L. plantarum-pentosus* T14 had higher cell numbers (10^{10} CFU/mL) than *L. plantarum-pentosus* T35 (10^9 CFU/mL) at 24 h fermentation. This suggests that differences in strains and sources of strains have different growth capabilities on the same medium. *L. plantarum-pentosus* T14 isolated from tempeh (soy fermented from Indonesia), so they are better able to adapt to grow in *kerandang* milk than *L. plantarum-pentosus* T35 isolated from bamboo shoot pickle. Mital and Steinkraus (1974) suggest that strain *L. acidophilus* ATCC No. 4356, *L. cellobiosis* NRRL-B-1840 and *L. plantarum* B-246 (10^9 CFU/mL) attained higher maximum populations in soymilk than *L. bulgaricus* (10^6 CFU/mL). In addition, Chun *et al.* (2007) explain that after 9 to 12 h of fermentation, cell population was the highest in soymilk inoculated with *L. paraplantarum* KM or *E. durans* KH than those with *S. salivarius* HM or *W. confusa* JY.

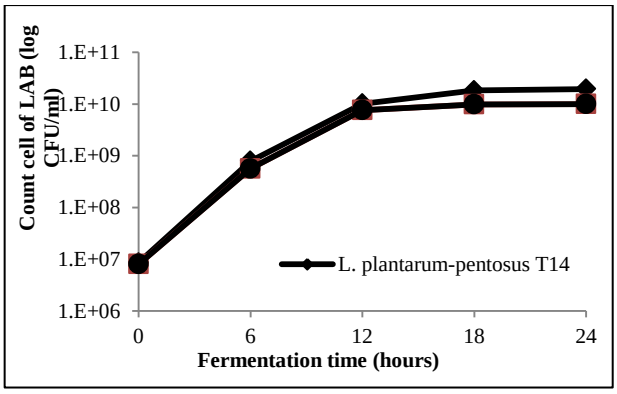

Figure 2: Growth of *L. plantarum-pentosus* T14 and *L. plantarum-pentosus* T35 in *kerandang* milk during fermentation at 37 °C.

Acid production and pH decline

Acid production expressed as lactic acid and change of pH during fermentation of *kerandang* milk at 37 °C is shown in Figure 3. Total acid expressed as total lactic acid increase during fermentation which followed by pH decrease. The decrease of pH of *L. plantarum-pentosus* T14 was more rapid during the first 12 h but *L. plantarum-pentosus* T35 was slower. The increase in total acid by *L. Plantarum-pentosus* T14 greater than that of *L. Plantarum-pentosus* T35. As well as in pH change, pH reduction by *L. Plantarum-pentosus* T14 greater than that *L. Plantarum-pentosus* T35. It is corresponded with bacterial growth and cell population, wherein the cell number of *L. plantarum-pentosus* T14 greater than *L. plantarum-pentosus* T35 (Figure 3). In accordance with Sumarna (2008), organic acid production, pH decline and other metabolic activities occurred during the first 12 to 24 h of incubation in soymilk, which corresponded to the exponential phase of the growth.

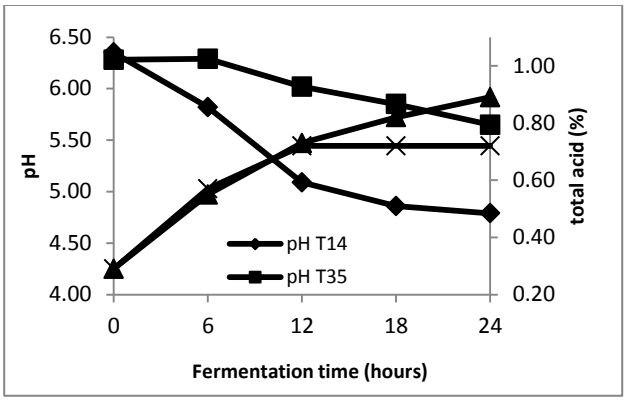

Figure 3: Acid production and change in pH of *kerandang* milk during fermentation at 37 °C by *L. plantarum pentosus* T14 and *L. plantarum pentosus* T35.

Saccharides metabolism in *kerandang* milk

L. plantarum-pentosus T14 and *L. plantarum-pentosus* T35 in this study were able to use raffinose for the growth. Utilization of raffinose by all strain are different, that showed by lactic acid production and decreasing of pH (final pH = 4.71-4.78) (Figure 3). The metabolisms of raffinose in *kerandang* milk varied and depend on the organism. The raffinose was metabolize by *Lactobacillus* into simple saccharides such as sucrose, glucose, fructose and galactose; and use it as an energy source for growth and lactic acid production by means of glycolysis pathway (Embden Meyerhof Pathway), this pathway represent the major means of glucose catabolism in most cells. This is shown by the residue of these saccharides in fermented *kerandang* milk (Table 2). The residue of raffinose in fermented *kerandang* milk by *L. plantarum pentosus* T14 is 38 ppm, sucrose 169 ppm, fructose 326 ppm, moreover glucose and galactose not detected. Whereas, the raffinose residue in fermented *kerandang* milk by *L. plantarum pentosus* T35 is 1098 ppm, sucrose 1373 ppm, fructose 282 ppm, moreover glucose and galactose not detected.

Table 2 shows the ability of *L. plantarum-pentosus* T14 to transform raffinose better than *L. plantarum-pentosus* T35. *Kerandang* milk contains raffinose 2151 ppm and during fermentation the raffinose was decrease. The raffinose transformation at 37 °C by *L. plantarum-pentosus* T14 are 68.59% for 12 h fermentation and 98.23% for 24 h fermentation, whereas *L. plantarum-pentosus* T35 are 35.25% for 12 h fermentation and 48.89% for 24 h fermentation. This is correlated with growth ability in *kerandang* milk which shown at Figure 2; the growth ability of *L. plantarum-pentosus* T14 (10^{10} CFU/mL) a high level than *L. plantarum-pentosus* T35 (10^9 CFU/mL) in the 24 h fermentation. *L. plantarum pentosus* T14 has the ability to transform raffinosa greater than *L. plantarum pentosus* T35. This is caused by two

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Table 2: Changes in concentration of raffinose, sucrose, glucose, fructose and galactose in *kerandang* milk during fermentation by *L. plantarum pentosus* T14 and *L. plantarum pentosus* T35 from Indonesia indigenous fermented foods at 37 °C.

Lactic acid bacteria strain	Saccharide	Saccharide concentration (ppm)				
		Incubation time (h)				
		0 (*kerandang* milk)	6	12	18	24
L. plantarum pentosus T14	Raffinose	2,152	1,416	676	488	38
	Sucrose	977	nd	290	778	169
	Glucose	nd	335	nd	392	nd
	Fructose	248	302	48	224	326
	Galactose	nd	nd	nd	nd	nd
L. plantarum pentosus T35	Raffinose	2,152	1,865	1,392	1,168	1,098
	Sucrose	977	1,402	1,579	783	1,373
	Glucose	nd	nd	nd	nd	nd
	Fructose	248	388	261	196	282
	Galactose	nd	nd	nd	nd	nd

nd: not detected

strains of different sources. *L. plantarum pentosus* T14 isolated from tempeh (fermented soy), so it is more able to adapt to grow in *kerandang* milk and metabolize raffinose into simple saccharides. Our findings are in line with those Sumarna (2008), who reported that fermentation of soymilk with *L. plantarum* SMN 25*, L. plantarum pentosus* SMN 01 and *L. plantarum pentosus* FNCC 235 reduced raffinose significantly (p<0.05) by 78.1%, 72.5% and 66%, respectively, whereas the remaining organisms showed less than 66% reduction after 24 h fermentation at 41 °C. Barampama and Simard (1994) has also reported that lactic fermentation by *Lactobacillus fermentum* ATCC 14931 reduce 97.28% raffinose in soaked-cooked dry bean of *Phaseolus vulgaris* var. Dore de Kirundo at 37 °C for 72 h fermentation.

CONCLUSION

Kerandang beans contain the saccharides (raffinose stachyose, sucrose and fructose) with the raffinose is the greatest. All two strains *L. plantarum-pentosus* T14 and *L. plantarum-pentosus* T35 can hydrolyze raffinose into simple saccharide and metabolize the saccharide during fermentation of *Kerandang* milk at 37 ºC for 24 h to produce lactic acid. The raffinose metabolism depends on strains of lactic acid bacteria. The raffinose metabolism by *L. plantarum-pentosus* T14 is 98.23% and *L. plantarum-pentosus* T35 is 48.89% in *Kerandang* milk. Thus, lactic fermented of *kerandang* milk will be safe for consumption.

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