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Fermentation of Jack Bean Milk Using Three Selected Lactic Acid Bacteria and Their Antioxidant Properties

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ABSTRACT: The aims of this research were to evaluate the ability of three strains of lactic acid bacteria as starter culture for jack bean milk fermentation and to study their chemical and functional properties. Fermentations were carried out at 37 °C for 24 h for each strain of *Lactobacillus plantarum* WGK 4, *Streptococcus thermophilus* Dad 11, and *Lactobacillus plantarum* Dad 13. Cell growth, titratable acidity (TA), pH, β-glucosidase activity, and total phenolic content (TPC) were monitored every six hours. Quantification of isoflavones was determined using Ultra Fast Liquid Chromatography (UFLC). The antioxidant properties were investigated using the 1,1-diphenyl-2-picrylhydrazyl (DPPH) method. The result showed that all three LAB strains grew well in jack bean milk to 9.15-9.26 log CFU/ml, produced acid, and decreased the pH from 6.54 to 4.71-5.00 after 24 h fermentation. During fermentation, all three LAB strains could produce β-glucosidase (27.08-27.18 mU/ml) and released aglycones content in jack bean milk (3.71- 4.02 µg/g daidzein and 9.00-10.26 µg/g genistein). TPC and DPPH radical scavenging activity increased 1.1-1.4 fold and 1.4 fold (30-42%), respectively. The results indicate all three LAB strains have a similar ability to increase antioxidant properties and potentially be good starter cultures for jack bean milk fermentation.

Keywords: antioxidant, jack bean milk fermentation, lactic acid bacteria

INTRODUCTION

Jack beans (*Canavalia ensiformis* (L) DC) are known as an underutilized legume and rich in protein (31.33%), carbohydrate (49.14%), minerals, and low in lipid (3.03%) (Sridhar and Seena, 2006; Puspitojati et al., 2020). As a legume family, jack beans might contain isoflavones, which play an important role in antioxidant radical scavenging activity (Djaafar et al., 2013; Koley et al., 2018). Mostly isoflavones in raw legumes appeared as isoflavone glycosides, which are difficult to absorb in the human intestine due to their high hydrophilicity and molecular mass (Gomez-Zorita et al., 2020). Isoflavone glycosides also have low antioxidant activity, caused by more hydroxyl groups attached to glucose molecules (Szeja et al., 2017; Krizova et al., 2019).

Like other legumes, jack beans might be processed into jack bean milk and also be fermented by lactic acid bacteria (LAB) to produce fermented jack bean milk. Numerous studies have shown that LAB fermentation in legume milk provides many benefits in improving functional compounds (Giyarto et al., 2009; Fitrotin et al., 2015). Fermentation of soymilk using five strains of lactobacilli was able to reduce the oligosaccharide content that caused flatulence in the digestive system (Singh and Vij, 2018). Sebastian et al. (2018) also reported that fermentation of soybean milk with cultures combination of *S. thermophilus* and *L. bulgaricus* resulted in increased antioxidant activity and changed amino acid composition. The ability of LAB to grow and change nutritional components during fermentation depends on the individual strain, medium composition, and fermentation time. For example, fermentation of soymilk using four different LAB strains (*L. plantarum* 00144, *L. delbrueckii* 01182, *B. breve* K-101, and *B. thermophilum* 00748) showed different patterns in cell growth and acid production during fermentation at 48 h (Pyo et al., 2005a). Fitrotin et al. (2015) demonstrated that different fermentation times significantly affected sesaminol triglucosides content and antioxidant properties in fermented sesame milk using *L. plantarum* Dad 13.

It has been known that some LAB could produce β-glucosidase to hydrolyze isoflavone glycosides into aglycones and glucose in legumes (Marazza et al., 2012; Hati et al., 2017). Pyo et al. (2005b) reported that the release of aglycones by β-glucosidase was correlated with

the enhanced antioxidant activity in fermented soymilk using *Bifidobacteria* strains. The correlation between the increase of antioxidant activity and the release of aglycones was also demonstrated in the fermentation of sesame milk with *L. plantarum* Dad 13 (Ulyatu et al., 2015), kerandang extract using five indigenous strains of *L. plantarum* (Djaafar et al., 2013), and soymilk with *B. longum* CRL849 (Marazza et al., 2013). Thus, the objective of this study was to investigate chemical and functional properties in jack bean milk fermented by three selected LAB strains *L. plantarum* WGK 4, *S. thermophilus* Dad 11, and *L. plantarum* Dad 13. The growth and acid production were also monitored during jack bean milk fermentation.

MATERIALS AND METHODS

Lactic acid bacteria and their cultivation

Lactobacillus plantarum WGK 4, *Streptococcus thermophilus* Dad 11, and *Lactobacillus plantarum* Dad 13 were obtained from the Food and Nutrition Culture Collection, Centre for Food and Nutrition Studies, Universitas Gadjah Mada, Yogyakarta, Indonesia. Cultures were maintained on MRS agar and stored at 4 °C in a refrigerator. The cultures were activated by growing the LAB individually in MRS broth twice at 37 °C for 24 h, and the resulting cultures served as culture starters.

Preparation of jack bean milk

Jack beans were obtained from Integrated Laboratory, Universitas Sarjanawiyata Tamansiswa, Yogyakarta, Indonesia. Jack beans were rinsed and soaked overnight in water with 0.25% NaHCO3 (1:5). The swollen jack beans were cooked, peeled, and extracted by a water ratio of 1:3 (w/v) using a blender (Phillips HR2116, Indonesia). The slurry was filtered through a double-layered cheesecloth, then transferred into sterile glass bottles for pasteurization at 65 °C for 20 min. The jack bean milk was cooled at room temperature before being inoculated with a starter culture.

Fermentation of jack bean milk

Each of the three selected LAB strains, *L. plantarum* WGK 4, *S. thermophilus* Dad 11, and *L. plantarum* Dad 13, was used as a starter culture for jack bean milk fermentation. The fermentation method was based on Yudianti et al. (2020) with some modifications. One hundred ml of jack bean milk was inoculated with 1 % (v/v) of activated cultures and incubated at 37 °C for 24 h. Samples were taken at 0, 6, 12, 18, and 24 h and were directly analyzed for viable cell numbers, titratable acidity, and pH. In the TPC and DPPH radical scavenging assay, the samples were extracted in methanol to produce a crude extract. For isoflavone determination, the samples were freeze-dried using a freeze-drier (Edward Modulyo, USA). Jack bean milk and 18 h fermented jack bean milk by each culture were determined the amount of sucrose, lactic acid and acetic acid contents.

Enumeration of viable cell

The pour plate method was used for the enumeration of viable populations of LAB using MRS agar containing $CaCO_3$. One milliliter of serial dilution samples was aseptically taken into plates and incubated at 37 °C for 48 h in the incubator (Sanyo MIR-262, Japan). The viable cells were expressed as CFU/mL.

Determination of titratable acidity and pH

Titratable acidity was determined by titration of samples with a 0.1 N NaOH solution using *phenolphthalein* as an indicator. The pH of the samples was monitored using a pH meter (Hanna Instruments HI 2210, USA) after calibrating with pH 4.0 and 7.0 standard buffers.

Sucrose analysis

Sucrose analysis was performed according to AOAC (1980). One gram of the freeze-dried sample was combined with 24 ml of deionized water and 1 ml of Correz reagent, then sonicated for 15 minutes using a sonicator (Eyela, Singapore). The mixture was centrifuged at 14,000 rpm for 3 min, and then the supernatant was filtered through a syringe filter GHP/RC 0.45 µm. Samples were analyzed with High Performance Liquid Chromatography (HPLC) in isocratic mode with Refractive Inject Detector (RID) and carbohydrate column (5 µm, 250 × 4.6 mm). Acetonitrile 80% was used as the mobile phase with 10 µl of sample injection and a flowrate of 1 ml/min.

Determination of lactic acid and acetic acid contents

Lactic acid and acetic acid content were determined according to a previous paper (Wasik et al., 2007). One gram of freeze-dried sample was added to 25 ml of 20 mM phosphate buffer, then sonicated for 10 min. The insoluble residue was separated by centrifugation at 4,000 rpm for 10 min. The step was continued by cleaning up using SPE-C18 and filtered through a syringe filter of 0.20 µm. The sample was analyzed using Ultra Performance Liquid Chromatography (UPLC) Acquity H Class system in the isocratic mode with photodiode array-detector (PDA) and C-18 column (100 x 3.0 mmI). The mobile phase was 20 mM phosphate with a flow rate of 0.425 ml/min, and the volume of injection was 10 µl.

Extraction and analysis of isoflavones

The extraction and analysis of isoflavones from the samples were performed using the method described by Sulistyowati et al. (2019). Two grams of freeze-dried sample was dissolved in 10 ml of HPLC-grade methanol 50% and sonicated using an ultrasonic cleaner (Eyela, Singapore) for 30 min at room temperature. Then, the mixture was centrifuged using a Thermo Fisher Scientific centrifuge at 3000 rpm for 15 minutes and filtered with Whatman paper no 1. The supernatant was collected and filtered using a 0.45 µm syringe filter (Minisart, USA). The samples were injected into the UFLC (Shimadzu 20A, Japan), with a photodiode array detector (UV-259 nm), C-18 column (5 µm, 150mm x 4,6mm), and a manual injector with 10 µl at 30 °C. Isoflavone aglycones were eluted by gradient runs of 15 min with mobile phase methanol (solvent A) and water containing 0.1% acetic acid glacial (solvent B) ratio 53:47 at a flow rate of 1 ml/min. Daidzein and genistein (Sigma-Aldrich, Singapore) (1-10 mg/ml) were used as standards.

Assay for β-glucosidase activity

The β-glucosidase activity was assayed by determining the rate of hydrolysis of nitrophenyl β-D-glucopyranoside (p-NPG) (Djaafar et al., 2013). The crude enzyme was prepared by centrifuging 10 ml samples at 4000 rpm (4 °C) for 15 min. The enzyme activity was determined by incubating mixtures of 1 ml of 5 mM substrate (p-NPG) prepared in 0.1 M sodium phosphate buffer (pH 7) and 0.5 ml crude enzyme at 37 °C for 30 min. The reaction was stopped by the addition of 1 ml of 1 M cold sodium carbonate (4 °C). The amount of p-nitrophenol released was measured using a spectrophotometer (Thermo Fisher Scientific, USA) at 401 nm. One unit of enzyme activity was defined as the amount of β-glucosidase that released 1 µmol of p-nitrophenol from p-NPG per ml per minute.

Preparation of crude extract

The crude extract was prepared according to Fitrotin et al. (2015) by dissolving two milliliters of the samples into 10 ml methanol 70%. The mixtures were shaken in a water bath shaker (Sibata, Japan) at 100 rpm (30 °C) for 72 min and macerated at 4 °C for 24 h. The crude extracts were obtained by centrifugation (Thermo Fisher Scientific, USA) at 3000 g (4 °C) for 15 min and filtration through Whatman paper no. 42. The extraction procedure was repeated twice to obtain the first and second supernatants. The supernatants were mixed and stored at -20 °C to determine phenolic content and DPPH radical scavenging activity.

Total phenolic content

The phenolic content (TPC) was determined using the Folin-Ciocalteu method, as reported Leonora et al. (2009) with slight modification. One milliliter of crude extract was diluted into 4 ml of distilled water, then 2 ml of the diluted samples or gallic acid solution was transferred into tubes and mixed with 1 ml of Folin-ciocalteu reagent. Sodium carbonate 15% was added into the mixture and kept in the darkroom for 2 h. The absorbance of the solution mixture was measured at 760 nm using a spectrophotometer (Thermo Fisher Scientific, USA) and gallic acid (0-25 mg/ml) was used as a standard. The results are expressed as gallic acid equivalents (GAE/100ml).

Free radical scavenging activity

Free radical scavenging activity was determined using DPPH radical, following the method of Marazza et al. (2012) with slight modification. One milliliter of the crude extract in every sample was added to 3 ml DPPH solution (0.1 mM), then incubated in the darkroom for 30 min. Absorbance was measured at 515 nm using a spectrophotometer (Thermo Fisher Scientific, USA). Methanol was used as a control, and ascorbic acid (0-10 mg/ml) was measured for comparison. The radical scavenging activity was calculated as follows:

$$\text{Radical Scavenging activity (\%)} = \left(\frac{(control - sample\ absorbance)}{(control\ absorbance}\right) \times 100\%$$

Statistical analysis

Data were analyzed by one-way ANOVA using SPSS 26 software. Results are expressed as mean ± standard deviation and considered significantly different with $P<0.05$. All data presented are mean values of 2 batch fermentations and 2 replicates of analysis (n=4).

RESULTS AND DISCUSSION

Fermentation of jack bean milk

The growth of LAB in jack bean milk during fermentation for 24 h at 37 °C is shown in Table 1. All three LAB strains showed a similar growth pattern, which increased the cell number by about 2 log cycles after 24 h of fermentation. They grew rapidly in the first 12 h of fermentation and then remained relatively constant until 24 h with a range of population 9.15- 9.26 log CFU/ml. The growth profiles were similar to the growth pattern of *L. plantarum* Dad 13 in milk (Wardani, 2017), five indigenous *L. plantarum* strains in kerandang (*Canavalia*

Table 1. The growth of selected LAB, titratable acidity, and pH during fermentation of jack bean milk at 37 °C for 24 h

Cultures/ fermentation time (h)		Viable cell (log CFU/ml)	Titratable acidity (%)	pH
L. plantarum WGK 4	0	$7.45^{a} \pm 0.02$	$0.43^{a} \pm 0.03$	$6.54^{d} \pm 0.04$
	6	$8.12^{b} \pm 0.33$	$0.50^{ab} \pm 0.08$	$5.98^{c} \pm 0.34$
	12	$8.99^{c} \pm 0.02$	$0.60^{b} \pm 0.03$	$5.34^{b} \pm 0.10$
	18	$9.29^{c} \pm 0.07$	$0.81^{c} \pm 0.11$	$4.85^{a} \pm 0.18$
	24	$9.26^{c} \pm 0.04$	$0.89^{c} \pm 0.07$	$4.71^{a} \pm 0.06$
S. thermophilus Dad 11	0	$7.76^{a} \pm 0.18$	$0.43^{a} \pm 0.03$	$6.54^{d} \pm 0.04$
	6	$8.19^{b} \pm 0.21$	$0.50^{a} \pm 0.08$	$6.06^{c} \pm 0.26$
	12	$9.08^{c} \pm 0.07$	$0.61^{b} \pm 0.02$	$5.27^{b} \pm 0.08$
	18	$9.20^{c} \pm 0.04$	$0.83^{c} \pm 0.09$	$5.07^{a} \pm 0.03$
	24	$9.25^{c} \pm 0.04$	$0.84^{c} \pm 0.02$	$5.00^{a} \pm 0.10$
L. plantarum Dad 13	0	$7.86^{a} \pm 0.00$	$0.43^{a} \pm 0.03$	$6.54^{d} \pm 0.04$
	6	$8.16^{b} \pm 0.24$	$0.52^{ab} \pm 0.11$	$5.96^{c} \pm 0.38$
	12	$9.05^{c} \pm 0.02$	$0.59^{b} \pm 0.08$	$5.28^{a} \pm 0.08$
	18	$9.12^{c} \pm 0.24$	$0.81^{c} \pm 0.06$	$5.04^{a} \pm 0.01$
	24	$9.15^{c} \pm 0.07$	$0.90^{c} \pm 0.09$	$5.00^{a} \pm 0.12$

Mean values in each culture with different superscripts are significantly different ($p<0.05$). Non-inoculated jack bean milk was written as 0 h.

virosa) extract (Djaafar et al., 2013), and *Bifidobacteria* in soymilk (Tsangalis et al., 2002). Meanwhile, Zhu et al. (2019) reported a lower number of viable cells in the fermentation of tofu whey by *L. plantarum* PL for 24 h (8.05 log CFU/ml) than those in our results.

Lactic acid bacteria use carbon, nitrogen sources, and other compounds from the medium for their growth and metabolic activity. It could be that these three strains of LAB could utilize sugars and other nutrients in jack bean milk. Jack bean milk contained 2.25% sucrose (Table 2), 0.35% fructose, and 0.52% maltose (data not shown). Doss et al. (2011) reported that jack bean contained oligosaccharides, such as raffinose (1.51%), stachyose (1.80%), and verbascose (4.86%). These sugars might be used as q sources for the growth and metabolic activity of lactic acid bacteria. *Lactobacillus plantarum* WGK 4, isolated from red lima bean soaking water in the production of tempe, could utilize sucrose, fructose, maltose, and raffinose (Yudianti et al., 2020), therefore, could use carbon sources in jack bean milk for their growth. It seems that *S. thermophilus* Dad 11 and *L. plantarum* Dad 13, which were isolated from Dadih, a fermented buffalo milk from West Sumatra, could also use carbon sources in jack bean milk for their growth, especially sucrose. No sucrose was detected after 18 h of fermentation of jack bean milk by these three LAB strains (Table 2). LAB also required amino acids such as glutamic acid, isoleucine, leucine, and valine, and minerals such as Mn^{2+}, Ca^{2+}, Fe^{2+}, K^{+}, and Na^{+} as their growth factors (Hayek and Ibrahim, 2013; Endo and Dicks, 2014). It has been reported that jack bean seeds contain a high amount of glutamic acid, aspartic acid, leucine, and proline (Puspitojati et al., 2020), also minerals, including Ca^{2+}, Mn^{2+}, Fe^{2+}, K^{+}, Na^{+}, Zn^{2+}, and Cu^{2+} (Sridhar and Seena, 2006). These compounds might help to stimulate the growth of LAB in jack bean milk.

These three strains not only grew well in jack bean milk but also produced acid, thereby reducing the pH (Table 1). All three selected LAB produced acids and lowered the pH in a similar pattern. There was a significant increase ($p<0.05$) in titratable acidity from 0.43% to 0.83-0.90%, in line with the decrease in pH from 6.50 to 4.71-5.00 during 24 h fermentation. These three LAB in jack bean milk produced lactic acid and acetic acid at 101.93-156.80 g/kg and 42.56-79.99 g/kg, respectively (Table 2). Ulyatu et al. (2015) reported that lactic acid and acetic acid were produced during 18 h of fermentation of sesame milk using *L. plantarum* Dad 13. Fermentation of soy milk by *L. plantarum* 001441 and *B. thermophilum* 00748 produced acid with titratable acidity of 0.81% and 0.79%, respectively, after 24 h of fermentation (Pyo et al., 2005a).

Generally, complex carbon sources were metabolized by LAB into simple sugars, such as glucose, fructose, and galactose, to obtain energy for their growth and metabolic activity via the Embden-Meyerhof pathway (Martinez et al., 2013). Since sucrose is the main fermentable sugar in jack bean milk, it could be that these LAB use sucrose for

their metabolic activities to produce acid. Our results showed that after 18 h of fermentation, sucrose were not detected in fermented jack bean milk (Table 2). Another possibility of obtaining fermentable sugars is from the hydrolysis of isoflavone glycosides to release isoflavone aglycone and glucose by β-glucosidase. Therefore, the activity of β-glucosidase during the fermentation of jack bean milk was monitored.

Table 2. Lactic acid, acetic acid, and sucrose content in jack bean milk and 18 h fermented jack bean milk using three selected LAB at 37 °C

Strains	Lactic acid (g/kg)		Acetic acid (g/kg)		Sucrose (%)	
	Initial	18 h	Initial	18 h	initial	18 h
L. plantarum WGK 4	ND	$113.09^{b} \pm 0.10$	ND	$57.11^{b} \pm 0.13$	2.25 ± 0.00	ND
S. thermophilus Dad 11	ND	$156.97^{c} \pm 0.13$	ND	$79.98^{c} \pm 0.25$	2.25 ± 0.00	ND
L. plantarum Dad 13	ND	$101.90^{a} \pm 0.11$	ND	$42.55^{a} \pm 0.03$	2.25 ± 0.00	ND

Mean values in the same row with different superscripts are significantly different ($p<0.05$). Non-inoculated jack bean milk was written as initial. ND (not detected).

β-glucosidase activity and isoflavone aglycones content

β-glucosidase activity during jack bean milk fermentation using three selected LAB is shown in Table 3. There was no β-glucosidase activity in unfermented jack bean milk. After 6 h of fermentation, the three selected LAB demonstrated their ability to produce β-glucosidase at a similar rate. The β-glucosidase activities increased during 6 h of jack bean milk fermentation using these three starter cultures. Following fermentation time, there was no significant increase in β-glucosidase activities with the values in the range of 27.08-27.17 mU/mL after 24 h fermentation. β-glucosidase activities were also detected in the fermentation of soymilk by *B. longum* CRL849 (39.6 UE/mL), and fermentation of sesame milk by *L. plantarum* Dad 13 (71.06 mU/mL) (Marazza et al., 2013; Fitrotin et al., 2015). Pyo et al. (2005a) reported that fermentation of soymilk with *L. delbrueckuu* lactic KFRI 01181 produced the highest β-glucosidase among the three other LAB strains and showed the most cell growth (9.1 log CFU/ml) at 24 h. Our study also found *L. plantarum* WGK 4, with the highest β-glucosidase activity (28.43 mU/ml), had the highest viable cell (9.29 log CFU/ml). It seems that β-glucosidase activity has a correlation with cell growth since hydrolysis of isoflavone glycoside by β-glucosidase releases isoflavone aglycone and glucose as fermentable sugar, thus, it will promote the growth of lactic acid bacteria and enhance the production of β-glucosidase. Based on scatter plot between viable cell vs enzyme activity, there were positif correlation between β-glucosidase activity and cell growth with r^2 values 0.67; 0. 58; and 0.55 for strains of WGK 4, Dad 11, and Dad 13 respectively Hati et al. (2020) reported that β-glucosidase production was also affected by other factors such as strains, type of ingredients, and culture conditions. β-glucosidase is an inducible enzyme that is synthesized when it is needed. The production of β-glucosidase is induced by its substrate in the lacks of a carbon source in the medium (Cairns and Esen, 2010). However, β-glucosidase activity has no significant difference ($p<0.05$) after 6 h fermentation because their end-product (glucose) inhibit their activity (Hati et al, 2020).

β-glucosidase plays a key role in hydrolyzing the β-glucosidic bond of isoflavone glycosides to release aglycones (Marazza et al., 2013). Isoflavone aglycones (daidzein and genistein) content during fermentation of the jack bean milk is shown in Table 3. The concentration of daidzein and genistein significantly increased during jack bean milk fermentation by all three LAB to 24 h and 6 h fermentation, respectively. There was no daidzein and genistein content detected in unfermented jack bean milk. Interestingly, these aglycones (daidzein and genistein) were released after 6 h of fermentation and followed the same pattern of β-glucosidase activity.

The final concentration of genistein at 24 h fermentation was 9.00-10.26 µg/g, approximately 2.2-2.5 fold higher than daidzein (3.71-4.02 µg/g). It indicates genistein contributed more to the total of aglycones concentration in jack bean milk. Marazza et al. (2012) reported a similar finding in fermented dry soymilk using *L. rhamnosus* CRL981 with genistein content 1.9-fold higher than daidzein. Xiao et al. (2015) reported a higher concentration of daidzein (5.15 mg/g) and genistein (4.68 mg/g) in fermented soy whey using *L. plantarum* B16

Table 3. β-glucosidase activity and the changes of the isoflavone aglycones concentration during fermentation of jack bean milk using selected LAB at 37 °C for 24 h

Culture/ fermentation time (h)		β-glucosidase activity (mU/mL)	Daidzein (µg/g)	Genistein (µg/g)
L. plantarum WGK 4	0	$0.00^{a} \pm 0.00$	$0.00^{a} \pm 0.00$	$0.00^{a} \pm 0.00$
	6	$26.86^{b} \pm 0.61$	$2.34^{b} \pm 0.03$	$9.56^{b} \pm 0.02$
	12	$28.43^{b} \pm 1.74$	$2.59^{b} \pm 0.02$	$9.32^{bc} \pm 0.06$
	18	$27.21^{b} \pm 1.28$	$3.46^{c} \pm 0.03$	$10.01^{bc} \pm 0.00$
	24	$27.13^{b} \pm 1.80$	$4.02^{d} \pm 0.02$	$10.26^{c} \pm 0.03$
S. thermophilus Dad 11	0	$0.00^{a} \pm 00.00$	$0.00^{a} \pm 0.00$	$0.00^{a} \pm 0.00$
	6	$27.20^{b} \pm 0.75$	$2.50^{b} \pm 0.02$	$9.14^{b} \pm 0.02$
	12	$27.80^{b} \pm 1.09$	$2.89^{c} \pm 0.03$	$9.76^{b} \pm 0.11$
	18	$27.56^{b} \pm 2.21$	$3.35^{d} \pm 0.03$	$9.20^{b} \pm 0.02$
	24	$27.17^{b} \pm 2.34$	$3.95^{e} \pm 0.02$	$9.00^{b} \pm 0.01$
L. plantarum Dad 13	0	$0.00^{a} \pm 0.00$	$0.00^{a} \pm 0.00$	$0.00^{a} \pm 0.00$
	6	$26.47^{b} \pm 0.95$	$2.33^{b} \pm 0.04$	$9.57^{b} \pm 0.01$
	12	$27.67^{b} \pm 1.37$	$3.31^{c} \pm 0.03$	$10.06^{b} \pm 0.06$
	18	$27.11^{b} \pm 2.85$	$3.38^{cd} \pm 0.01$	$9.84^{b} \pm 0.07$
	24	$27.08^{b} \pm 2.43$	$3.71^{d} \pm 0.03$	$9.28^{b} \pm 0.01$

Mean values in each culture with different superscripts are significantly different ($p<0.05$). Non-inoculated jack bean milk was written as 0 h.

compared to our results. However, aglycone content in fermented kerandang (*Canavalia virosa*) extracts using five indigenous *L. plantarum* reported had lower results than our study, resulting in 1-2 µg/g of daidzein and 0.5 µg/g of genistein (Djaafar et al., 2013). The differences in the release of aglycones during fermentation were affected by the activity of β-glucosidase produced by each lactic acid bacteria and also the availability of carbon source and isoflavone glycoside as its inducer.

Hati et al. (2015) found that the increase of aglycone content accompanied by the decrease of isoflavone glycosides in the fermentation of soymilk using *L. rhamnosus* C6 at 12 h. Farianti et al. (2015) also reported the same finding in the fermentation of kerandang (*Canavalia virosa*) extract using *L. plantarum-pentosus* T-14 at 24 h. Furthermore, Zhao and Shah (2014) mentioned that the content of aglycones was associated with total phenolic content and antioxidant activity. Hydrolysis of isoflavone glycosides by β-glucosidase produces isoflavone aglycones and glucose. Glucose is easily utilized by lactic acid bacteria for their growth and metabolic activity. Therefore β-glucosidase activity provides fermentable sugar for lactic acid bacteria during jack bean milk fermentation and might increase antioxidant activity.

Total phenolic content (TPC)

TPC in fermented jack bean milk using LAB is shown in Table 4. All three selected LAB showed a similar ability to release free phenolic content and significantly increased ($p < 0.05$) along with 24 h fermentation. TPC of unfermented jack bean milk was 76.60 mg GAE/100 ml, then increased to 81.02-85.37 mg GAE/100 ml (1.1 fold) at 24 h fermentation. A similar pattern was described by Ulyatu et al. (2015) in fermented sesame milk using *L. plantarum* Dad 13, the increase of phenolic content was 2.1-fold higher after 18 h of fermentation. Xiao et al. (2015) also reported a similar range to our study by increasing total phenolic (1.2 fold) in fermented soy whey extracts using *L. plantarum* B1-6 at 24 h. In addition, Lee et al. (2018) found higher results of the change in phenolic content from 2.4 to 3.6 mg GAE/g (1.5 fold) in fermented soy-powder using *L. plantarum* S48.

Table 4. Total phenolic and radical scavenging activity during fermentation of jack bean milk using selected LAB at 37 °C for 24 h

Culture/ fermentation	Time (h)	Total phenolic (mg GAE/100mL)	Radical scavenging activity (%)
L. plantarum WGK 4	0	$76.60^{a} \pm 0.00$	$20.98^{a} \pm 2.18$
	6	$79.10^{b} \pm 0.00$	$22.02^{ab} \pm 0.14$
	12	$80.55^{c} \pm 0.08$	$23.05^{cd} \pm 0.63$
	18	$83.52^{d} \pm 0.08$	$24.54^{d} \pm 0.15$
	24	$84.05^{e} \pm 0.00$	$27.47^{e} \pm 0.07$
S. thermophilus Dad 11	0	$76.60^{a} \pm 0.00$	$20.98^{a} \pm 2.18$
	6	$79.10^{b} \pm 0.00$	$22.57^{ab} \pm 0.21$
	12	$80.16^{c} \pm 0.30$	$24.33^{b} \pm 0.14$
	18	$82.47^{d} \pm 0.23$	$24.82^{b} \pm 0.00$
	24	$85.37^{e} \pm 0.23$	$28.97^{c} \pm 2.81$
L. plantarum Dad 13	0	$76.60^{a} \pm 0.00$	$20.98^{a} \pm 2.18$
	6	$77.43^{b} \pm 0.30$	$24.33^{ab} \pm 0.42$
	12	$78.91^{c} \pm 0.69$	$25.06^{b} \pm 0.14$
	18	$80.03^{d} \pm 0.46$	$26.40^{b} \pm 0.76$
	24	$81.02^{e} \pm 0.08$	$29.82^{c} \pm 0.34$

Mean values in each culture with different superscripts are significantly different ($p<0.05$). Non-inoculated jack bean milk was written as 0 h.

Generally, phenolic compounds in food are available in multiple forms, including soluble free compounds, soluble conjugated to sugars and other low molecular mass components, and insoluble bound form (Zhang et al., 2012). From those descriptions, isoflavone glycosides were classified as phenolic conjugated, which might release free phenolic aglycones during LAB fermentation and increase the TPC in jack bean milk. This assumption was supported by Lee et al. (2018) and Lai et al. (2013) that the increase of TPC in their studies was caused by the change of conjugated phenolic type into free phenolic, which was catalyzed by β-glucosidase. Niveditha and Sridhar (2014) found a correlation among TPC, β-glucosidase, and antioxidant capacity in fermented beans of *Canavalia* with *Rhizopus oligosporus*. Besides that, other phenolic compounds also bonded to macromolecule components such as carbohydrates, proteins, and lipids (Zhang et al., 2014), which might contribute to the increase in phenolic content during fermentation. Hur et al. (2014) mentioned the transformation of phenolic compounds related to enzyme production by LAB, such as glucosidase, amylase, cellulase, invertase, or lipase, to hydrolyze glucosides and break down the cell plant walls. Moreover, Chandrasekara and Shahidi (2012) exhibited that phenolic compounds bonded to soluble fiber were released during microbial fermentation.

Free radical scavenging activity (RSA)

Radical scavenging activity in fermented jack bean milk using LAB was determined by the DPPH method, shown in Table 3. All three selected LAB had a similar ability to significantly increase antioxidant activity ($p<0.05$) by the time of fermentation for 24 h. Radical scavenging activity of unfermented jack bean milk was 20.98% and enhanced during 24 h fermentation, reaching values 27.47-29.82% (1.3-1.4 or 35-40% higher than unfermented jack bean milk). Compared to % RSA in ascorbic acid, those results were equal to more than 6 mg/L (22.37%) but less than 8 mg/L (31.73%) (data not shown). Fitrotin et al. (2015) found a higher increase of % RSA in the fermentation of sesame milk using *L. plantarum* Dad 13 (2.3 fold) and Marazza et al. (2013) in soymilk with *B. longum*

CRLL849 (3.2 fold). Meanwhile, our results were in range with the fermentation of kerandang extract (*Canavalia virosa*) using five *L. plantarum* strains, which obtained %RSA 1.33 to 2 times at 24 h (Djaafar et al., 2013).

Antioxidant activity is the capacity of antioxidant compounds to eliminate free radicals in the cell or food (Hur et al., 2014). Several studies showed that antioxidant capacity was associated with phenolic compounds such as flavonoids, phenolic acid, and tannins (Jin et al., 2012). This finding was in line with our study, which showed that the increase in % RSA during fermentation was followed by the rise of total phenolic content. It has been reported that isoflavone conversion is related to the enhancement of antioxidant activity in the fermented legume (Pyo et al., 2005b; Djaafar et al., 2013; Hati et al., 2020). Pyo et al. (2005b) found a high correlation between antioxidant activity and aglycones production during the fermentation of soybean *Lactobacillus* and *Bifidobacteria* (R=0.8299) for 24 h. This relation involved a hydroxyl group addition in atom C-7 of aglycones released from the hydrolysis of isoflavone glycosides by β-glucosidase (Tsangalis et al., 2002; Otieno et al., 2005). Marazza et al. (2013) explained that the power to scavenge free radicals in isoflavones was also related to the number and location of aromatic hydroxyl groups. For example, genistein presents three hydroxyl groups, while daidzein only has two. The different number of total hydroxyl groups in genistein has a higher ability to scavenge radicals than daidzein. On the other side, antioxidant activity might not only be from the increase of aglycone content in fermented jack bean milk. Several studies have proven that LAB increases other antioxidant substances during fermentation, such as chelating agents, enzymatic antioxidants, and bioactive peptides (Hur et al., 2014; Suo et al., 2016; Puspitojati et al., 2020). Suo et al. (2016) found fermentation of soybean milk with *L. fermentum* increased free radical scavenging activity of reactive oxygen species, hydroxyl radical, and catalase activity. Puspitojati et al. (2020) also reported that jack bean tempe contained high bioactive peptides after fermentation with *Rhizopus microspores*, *R. oligosporus*, and *R. rhizopodiformis*. Based on those references, another antioxidant examination might be needed to understand all compounds involved in the antioxidant properties of fermented jack bean milk.

CONCLUSION

Three selected LAB could grow well in jack bean milk and produced acid. They showed similar patterns of cell growth, acid production, and pH value during the fermentation of jack bean milk. During jack bean milk fermentation, lactic acid bacteria produced β-glucosidase, which hydrolysed isoflavone glycoside to release aglycones content, increased free phenolics, and enhanced DPPH radical scavenging activity at a similar rate. All three selected LAB seem to be promising strains for improving the antioxidant properties in jack bean milk through fermentation.

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