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SINGLE AND MIXED LACTIC ACID BACTERIA CULTURE FERMENTATION IN RED BEAN MILK FOR DEVELOPMENT OF A FUNCTIONAL BEVERAGE

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ABSTRACT

This study was to evaluate the effect of fermentation on the total phenolic contents (TPCs) and antioxidant activities (AA) in red bean milk (RBM) using three different lactic acid bacteria (LAB); *Streptococcus thermophilus* TISTR 894 (*ST*), *Lactobacillus plantarum* 299V (*LP*), and *Lactobacillus casei* 388 (*LC*), as a single (SCF) or a mixed culture fermentation (MCF). The fermentation increased TPCs value and AA values in the RBM, irrespective of fermentation technique or LAB cultures. Among SCF, the SCF-*LP* showed the highest values of TPCs, DPPH, and FRAP while SCF-*LC* had the highest ORAC assay values. The MCF could lead to higher TPCs and AA than those of SCF. Especially, MCF using three cultures of LAB presented the highest TPCs, DPPH, and FRAP assay (1.47 mg GAE/g DW, 11.59 μmole TE/g DW, and 4.89 μmole TE/g DW, respectively). Moreover, the study showed that the MCF can effectively improve the TPCs and AA, thereby indicating additional health benefits of fermentation.

Key words: Antioxidant activities, lactic acid bacteria, mixed culture fermentation, red bean, total phenolic content

INTRODUCTION

Lactic acid bacteria (LAB) have also been traditionally used for legume fermentation because they are naturally present in legumes. Many evidences showed that the fermentation of legumes with LAB strains, such as those of *Lactobacillus* genera, improved favor, produce bioactive compounds which provide health benefits beyond basic nutrition (Limón *et al*., 2015; Savijoki *et al*., 2006). During the fermentation, LAB hydrolyzed nutrition and phytochemical compounds (Tomovska *et al.*, 2013), leading to increasing of the antioxidative capacity that benefits to human health (Gjorgievski *et al*., 2014).

The mixed culture fermentation attempts to shorten the fermentation time, reduce microbial contaminate and provides complex metabolites that can also considerably increase the functional properties of foods. Also, the increase of AA via the mixed cultures fermentation was demonstrated (Jhan *et al*., 2015). To our knowledge, there is no information available regarding the effect of mixed culture fermentation by using probiotic bacteria on the total phenolic compounds and antioxidant activities in red bean milk. Thus, the objective of this work was to determine the effect of single or mixed culture fermentation on the total phenolic contents and antioxidant activities in red bean milk. by using *Lactobacillus casei* 388, *Lactobacillus plantarum* 299V and *Streptococcus thermophilus* TISTR 894. In addition, results derived from this work would provide valuable information on the bioactivity compounds of red bean fermented ingredients in to develop novel functional foods.

MATERIALS AND METHODS

Red bean milk preparation

Red bean powder (RBP) was sieved through a 100-mesh sieve. Xanthan gum 0.7 g was mixed with deionized water 100 mL at temperature of 60°C. After that 10 g of RBP and 10 g of refined sugar

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were added and mixed ingredients until homogeneous. Then the red bean milk was pasteurized at 100°C for 30 minutes and cooled to room temperature prior inoculation.

Starter culture preparation

Lactobacillus casei 388 (*LC*), *Lactobacillus plantarum* 299v (*LP*) and *Streptococcus thermophilus* TISTR 894 (*ST*) were were steaked on media agar and incubated at 37°C for 24 hr to obtain the selected single colony. After that one colony were inoculated into MRS broth to activate at 37°C for 18-20 hr.

Single culture (SCF) and mixed cultures fermentation (MCF) of red bean milk

From MRS broth, the cultures were washed twice with deionized water. Then, each culture was inoculated to the fermentation with an initial population of 6.00 log CFU/mL to pasteurized red bean milk. The mixed culture with two or three cultures were initially inoculated with 5.70 log CFU/mL and 5.52 CFU/mL of each pasteurized red bean milk. Then, the samples were incubated at 37°C for 12 hours. strain, respectively.

Fermented red bean milk extraction

The freeze dried samples of red bean milk and FRBM with approximate amount of 0.30 grams dry weight were extracted with deionized water. The samples were extracted twice with deionized water and then centrifuged at 4°C, 4600 rpm for 15 min. After that, only supernatant was filtered through FILTREX cellulose acetate syringe filter (0.22 μm) and kept in the dark place at 20°C for further analysis.

Determination of total phenolic contents (TPCs)

The TPCs of FRBM extracts were determined by Folin-Ciocalteu method (Ainsworth & Gillespie, 2007). The extracts of 25 μL were mixed thoroughly with 50 μL of 10% (v/v) Folin-Ciocalteu reagent and then incubated for 5 min at 25°C. The 200 μL sodium carbonate (7.5% w/v) were added to each well and reacted in the dark place at 25°C for 2 hr. The reaction was measured at 765 nm using a microplate reader (SynergyTM HT 96-well UV-visible spectrophotometer, BioTek Instruments, Inc., Winooski, VT).

Determination of antioxidant activities (AA)

1,1–diphenyl–2–picrylhydrazyl (DPPH) free radical scavenging activity

The 22 μL of FRBM extracts were mixed with 200 μL of DPPH solution that prepared by 150 μM DPPH in 95% ethanol, then kept in the dark place at room temperature for 30 min, and absorbance was measured at 520 nm.

Ferric reducing antioxidant power (FRAP) assay

The FRAP reagent was prepared from 300 mM of acetate buffer (pH 3.6), 10 mM TPTZ in 40 mM HCl and 20 mM $FeCl_3.6H_2O$ in the ratio of 10:1, kept in 37°C before used. The FRBM extracts (20 μL) were mixed with FRAP reagent and incubated in the darkroom for 8 min at room temperature. The samples were measured at 595 nm using the microplate reader (Benzie & Strain, 1996).

Oxygen radical absorbance capacity (ORAC) assay

The FRBM extracts (25 μL) were mixed with 150 μL of Fluorescein solution that prepared by 244 μL of 4.19 fluorescein solution to 12.5 mL with ORAC buffer and After that, it was kept in the dark place at 37°C for 30 min, then mixed with 153 mM APPH 25 μL. The samples were measured at absorbance 485 nm for excitation wavelength and 528 nm (Ou *et al*., 2001).

Statistical analysis

Three replications were performed for each experiment and the results were expressed as mean ± SD. Data were analyzed by using analysis of variance in the SPSS program. The significant differences between treatments were determined with the Duncan's test at the $p < 0.05$ level.

RESULTS AND DISCUSSION

Determination of total phenolic contents (TPCs)

The TPCs of fermented red bean milk (FRBM) showed in Table 1. The TPC values of red bean milk were in the range of 0.85 to 0.99 mg GAE/g DW. TPCs was increased approximately 2-folds after the fermentation. The TPC values of FRBM were in the range of 1.14 to 1.47 mg GAE/g DW. Among single culture fermentation (SCF), SCF-*LP* had the highest TPC value of 1.32 mg GAE/g DW Comparing to SCF, mixed culture fermentation (MCF) had a significantly higher content ($p < 0.05$) of TPCs value. A maximum TPCs value observed in MCF-CPS and MCF-CP with the value of 1.47 and 1.45 mg GAE/g DW, respectively.

Some studies explained that the fermentation improved TPCs (Sandhu *et al*., 2017) by increasing the release phenolic compounds presented in red bean such as sinapic acid, p-coumaric acid, ferulic acid, pelargonidin, kaempferol 3-O-glucoside, and cyanidin-3-O-4-glucoside (Lin *et al*., 2008). The fermentation could increase bioavailability and affect phenolic compounds depending on their

Table 1. Value of total phenolic contents of fermented red bean milk with LAB after fermentation

Fermented treatments	Total phenolic contents (mg GAE/g DW)	
	Initial	Final
SCF-*LC*	0.99±0.16^{Aa}	1.28±0.07^{ABb}
SCF-*LP*	0.85±0.14^{Aa}	1.32±0.03^{ABb}
SCF-*ST*	0.89±0.08 ^{Aa}	1.14±0.12^{Ab}
MCF-*CP*	0.99±0.14 ^{Aa}	1.45±0.11^{Bb}
MCF-*CS*	0.91±0.12 ^{Aa}	1.34±0.07^{Bb}
MCF-*PS*	0.97±0.16^{Aa}	1.36±0.05^{Bb}
MCF-*CPS*	0.95±0.08 ^{Aa}	1.47±0.11^{Bb}

The values were presented as mean ± SD of three replications.
a – b Values in the same row with different uppercase superscript letters were analyzed with T-test comparing with initial (red bean milk) and final (after fermented 12 hr).
A-B Values in the same column with different uppercase superscript letters were analyzed with one-way ANOVA. Means with different letters indicate significant differences ($p < 0.05$) comparing between the strain of probiotics fermented product by the initial and the end of fermentation.

Table 2. Value of antioxidant activities were analyzed by DPPH radical scavenging assay of fermented red bean milk with LAB after fermentation

Fermented treatments	DPPH (μmole TE/g DW)	
	Initial	Final
SCF-*LC*	3.68±0.33^{Aa}	6.32±0.41^{ABb}
SCF-*LP*	3.81±0.54^{Aa}	7.09±0.61^{ABb}
SCF-*ST*	3.68±0.54^{Aa}	5.27±0.19^{Ab}
MCF-*CP*	3.83±0.14^{Aa}	9.95±0.21^{CDb}
MCF-*CS*	3.91±0.46^{Aa}	8.83±0.54^{BCb}
MCF-*PS*	3.96±0.60^{Aa}	9.82±0.79^{CDb}
MCF-*CPS*	3.79±0.60^{Aa}	11.59±0.20^{Db}

The values were presented as mean ± SD of three replications.
a – b Values in the same row with different uppercase superscript letters were analyzed with T-test comparing with initial (red bean milk) and final (after fermented 12 hr).
A-D Values in the same column with different uppercase superscript letters were analyzed with one-way ANOVA. Means with different letters indicate significant differences ($p < 0.05$) comparing between the strain of probiotics fermented product by the initial and the end of fermentation.

transformation by specific components of the fermentative microbiota via esterase, glucosidase, dehydroxylase, and decarboxylase activities (Selma *et al*., 2009). During fermentation, the micro-organism could hydrolyze complexes of phenolic into soluble-free phenols and more active biological compounds that were readily to metabolite by proteolytic enzymes (Ademiluyi & Oboh, 2011; Adetuyi & Tesleem, 2014). In addition, the survival and synergistic growth of mixed cultures might assist to hydrolyze nutrition in red bean milk to amino acids, short chain fatty acids, and organic acids during fermentation.

Determination of antioxidant activities (AA)

1,1–diphenyl–2–picrylhydrazyl (DPPH) radical scavenging assay

The result of the DPPH radical scavenging assay of FRBM is shown in Table 2. The DPPH radical scavenging values of red bean milk before the fermentation were in range between 3.68 to 3.96 μmole TE/g DW. After the fermentation, the DPPH radical scavenging values in FRBM were significantly increased to 5.27 to 11.59 μmole TE/g DW. Among the FRBM with single culture, SCF-*LP* presented the maximum values in DPPH radical scavenging values ($p < 0.05$) of 7.09 μmole TE/g DW. In addition, the MCF with three strains exhibited the most DPPH radical scavenging values among all the fermentations ($p < 0.05$) while two LAB cultures fermentation presented the highest DPPH radical scavenging values of 9.95 μmole TE/g DW in MCF-CP. A similar finding showed that the AA of ABTS, DPPH, and FRAP markedly increased when used MCF of *LC* and *LP* in the fermented tomato juice (Liu *et al*., 2018). The study explained that *LP* represents potent AA for hydroxyl, and DPPH radical scavenging and for reducing power assays.

The MCF might increase the ability to hydrolyzed polysaccharide (Andersson *et al*., 2011). It might be a favorable factor for its higher AA (Huang *et al*., 2013; Zhao *et al*., 2018). Some researchers also suggested a correlation between DPPH radical scavenging activity and exopoly-saccharide, in which lower molecular weight polysaccharide might increase antioxidant activities (Liu *et al.,* 2010; You *et al*., 2013). Consequently, all these results showed that the DPPH radical scavenging values produced by the mixed culture fermentation had high potential as natural for bioactive additive in the food product (Yerlikaya *et al*., 2013).

Ferric reducing antioxidant power (FRAP) assay

The FRAP values were presented in Table 3. The value of red bean milk before the fermentation was in the range of 2.06 to 2.80 μmole TE/g DW and significantly increased to 3.08–4.89 μmole TE/g DW after the fermentation. SCF-*LP* had the highest FRAP value of 3.61 μmole TE/g DW when compared to other SCF. Moreover, this study illustrated that the FRAP value of MCF was higher than SCF after fermentation for 12 hr at 37°C. All of two culture fermentations, the MCF-CP had the highest FRAP value of 4.857 μmole TE/g DW. The mixed culture fermentation with three strains had the greatest FRAP value of 4.89 μmole TE/g DW.

Table 3. Value of antioxidant activities were analyzed by FRAP assay of fermented red bean milk with LAB after fermentation

Fermented treatments	FRAP (μmole TE/g DW)	
	Initial	Final
SCF-*LC*	2.42±0.07^{Aa}	3.56±0.11^{ABb}
SCF-*LP*	2.38±0.09^{Aa}	3.61±0.07^{ABb}
SCF-*ST*	2.06±0.16^{Aa}	3.08±0.17^{Ab}
MCF-*CP*	2.80±0.12^{Aa}	4.85±0.31^{Cb}
MCF-*CS*	2.54±0.08^{Aa}	3.89±0.07^{Bb}
MCF-*PS*	2.45±0.16^{Aa}	3.74±0.18^{Bb}
MCF-*CPS*	2.73±0.14^{Aa}	4.89±0.58^{Cb}

The values were presented as mean ± SD of three replications.
a – b Values in the same row with different uppercase superscript letters were analyzed with T-test comparing with initial (red bean milk) and final (after fermented 12 hr).
A-C Values in the same column with different uppercase superscript letters were analyzed with one-way ANOVA. Means with different letters indicate significant differences ($p < 0.05$) comparing between the strain of probiotics fermented product by the initial and the end of fermentation.

Table 4. Value of antioxidant activities were analyzed by ORAC assay of red bean milk fermented with LAB after fermentation

Fermented treatments	ORAC (μmole TE/g DW)	
	Initial	Final
SCF-*LC*	5.44±0.08^{Aa}	8.75±0.24^{ABb}
SCF-*LP*	5.01±1.06^{Aa}	8.27±0.24^{Ab}
SCF-*ST*	5.76±0.88^{Aa}	7.98±0.14^{Ab}
MCF-*CP*	5.92±2.36^{Aa}	9.72±0.51^{Bb}
MCF-*CS*	5.84±0.81^{Aa}	8.75±1.21^{ABb}
MCF-*PS*	5.99±1.00^{Aa}	11.76±0.31^{Cb}
MCF-*CPS*	5.62±0.27^{Aa}	11.39±0.59^{Cb}

The values were presented as mean ± SD of three replications.
a – b Values in the same row with different uppercase superscript letters were analyzed with T-test comparing with initial (red bean milk) and final (after fermented 12 hr).
A-C Values in the same column with different uppercase superscript letters were analyzed with one-way ANOVA. Means with different letters indicate significant differences ($p < 0.05$) comparing between the strain of probiotics fermented product by the initial and the end of fermentation.

Many studies had demonstrated that the microbial fermentation enhanced FRAP value when compared to the legume samples (Qin *et al*., 2010; Adetuyi & Ibrahim, 2014). They also explained that the increased FRAP activity might be related to the release of chelated iron compounds during the fermentation of plant-based products, for example, the fermented legume products with *Bacillus* spp. Similarly, the fermented soymilk and mung milk with *LP* showed the increase of FRAP values after the fermentation (Gan *et al*., 2016).

Oxygen radical absorbance capacity (ORAC) assay

The ORAC values (Table 4) were significantly increased from 5.01–5.99 μmole TE/g DW to 7.98-11.76 μmole TE/g DW after the fermentation ($p < 0.05$). The greatest value on SCF was SCF-*LC* (8.750 μmole TE/g DW). The MCF exhibited greater ORAC values than the SCF, especially MCF-PS that performed the highest amount of ORAC values of 11.76 μmole TE/g DW. According to Martinez-Villaluenga *et al*. (2012) presented that vegetable fermentation with LAB led to the increase of ORAC values by attributed to vitamin and polyphenols in product (Sikora *et al*., 2008). *LC* has been reported that it had high ability to neutralize 2,2'-Azobis (2-amidinopropane) dihydrochloride (AAPH) derived peroxyl radicals and lipophilic radicals derived from lipid peroxidation of products, which suggested that the presence of lipophilic antioxidants could protect against induced-free radicals on products (Takebayashi *et al*., 2010). Similar result by Chiu *et al*. (2013), the fermented pepino milk products with *LC* had higher ORAC values than either pepino milk product or the fermentation with other strains.

A previous study demonstrated that probiotic bacteria in MCF could be assisted in producing organic acids, which these molecules are an excellent electron donor. Thus, the MCF might improve AA by increasing the release of phytochemical compounds from plant-based foods (Đorđević *et al*., 2010).

In summary, the LAB fermentation in this study present significantly enhanced the antioxidant activities, irrespective of fermentation techniques or the culture used. Whereas, the FRBM with MCF can promote higher TPCs and AA than those of SCF. Although the DPPH and FRAP assay of FRBM with SCF-*LP* had the highest values compared with SCF, ORAC assay of SCF-*LC* had the highest values when compared with SCF. MCF-CPS had the highest value of DPPH, and FRAP assay, while MCF-PS had the maximum ORAC assay value, compared with MCF with two cultures used. These different assays to measure can be described as the reaction of antioxidants that interact electron free radicals through a different mechanism, including hydrogen atom transfer (HAT) or single electron transfer mechanism (SET) or the combination of both HAT and SET mechanism. In HAT mechanism, the ability of the free radical to remove one hydrogen atom from antioxidant or hydrogen donation is measured in SET mechanism. The ability of a potential antioxidant to transfer one electron to reduced compounds, including metals, carbonyls, and radicals is determined DPPH radical scavenging is

the assay based on SET and HAT mechanisms. Whereas FRAP utilizes the SET reaction and ORAC utilizes the HAT reaction. Hence, in this study presented FRBM with MCF-CPS had the highest value of TPCs, DPPH, and FRAP assay comparing of all samples, but ORAC assay presented FRBM with MCF-PS had the maximum values of all samples. Besides, LAB cultures could be led to variation of AA mechanism (Virtanen *et al*., 2007). However, the antioxidants in FRBM might be able to donate hydrogen atom and transfer the single electron because all AA values were increased after fermentation both of SCF and MCF.

CONCLUSION

A non-dairy fermented red bean milk beverage is a promising product with potential functional properties and would be beneficial to consumer health. The fermentation with LAB strain, including *LC*, *LP*, and *ST* is a benefit to future fermented products. Wherewith, they can produce TPCs and antioxidant activity (AA) values. Moreover, TPCs and AA values of the mixed culture fermentation were increased, especially the MCF with three LAB strains. From this study, it can be highlighted the use of mixed culture fermentation to enhance bioactive components and potential AA which improves their health-linked functionality in non-dairy fermented products. Therefore, this study was developed as imparting of knowledge for a functional beverage in novel foods by using mixed culture fermentation technique in non-dairy base substrate.

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