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Antioxidant Activity of Isoflavone Aglycone from Fermented Black Soymilk Supplemented with Sucrose and Skim Milk Using Indonesian Indigenous Lactic Acid Bacteria

Benediktus Yudo Leksono[1], Muhammad Nur Cahyanto[1], Tyas Utami [1,2*]

1-Department of Food and Agricultural Product Technology, Faculty of Agricultural Technology, Universitas Gadjah Mada, Yogyakarta, Indonesia

2- Center for Food and Nutrition Studies, Universitas Gadjah Mada, Yogyakarta, Indonesia

Abstract

Background and Objective: Black soybean (*Glycine max* (L.) Merr.) Detam-1 variety includes high quantities of isoflavone majorly in glucoside form; in which, the biochemical antioxidant activity was lower than that in isoflavone aglycone form. Fermentation by lactic acid bacteria can increase antioxidant activity and isoflavone aglycone of black soymilk. However, the biochemical ability is strain-dependent and sucrose or skim milk supplementation during processing may affect this ability. The objective of the current study was to investigate antioxidant properties of the fermented black soymilk and fermented black soymilk supplemented with 2% sucrose or skim milk using three Indonesian indigenous lactic acid bacteria, namely *Lactobacillus plantarum* WGK 4, *Streptococcus thermophilus* Dad 11 and *Lactobacillus plantarum* Dad 13. Furthermore, cell growth and acid production were investigated.

Material and Methods: Fermentation of black soymilk and black soymilk supplemented with 2% sucrose or skim milk was carried out using three indigenous lactic acid bacteria at 37 °C for 18 h. Viable cell, pH, titratable acidity, β-glucosidase activity, isoflavone aglycone, total phenolic content and antioxidant activity of black soymilk were assessed at the beginning of the experiment and after 18 h of fermentation.

Results and Conclusion: Results showed that all strains could grow (9 log CFU ml^{-1}) and produce acid in black soymilk and black soymilk supplemented with 2% sucrose or skim milk. Fermentation increased isoflavone aglycone through β-glucosidase activity, which resulted in increased total phenolic content and antioxidant activity. Fermented black soymilk with no sucrose or skim milk exhibited the highest β-glucosidase activity (19.66-21.54 mU ml^{-1}), daidzein formation (62-74%), genistein formation (67-80%) and antioxidant capacity (32.81-38.47%). All three lactic acid bacteria strains enhanced antioxidant activity and isoflavone aglycone of the black soymilk. Sucrose or skim milk addition did not affect the cell growth but increased acid production and decreased β-glucosidase activity and isoflavone aglycone formation. These three lactic acid bacteria included similar abilities to enhance antioxidant activity and isoflavone aglycone formation in fermented black soymilk.

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***Corresponding author:**

Tyas Utami*
Department of Food and Agricultural Product Technology, Faculty of Agricultural Technology, Universitas Gadjah Mada, Yogyakarta, Indonesia
Center for Food and Nutrition Studies, Universitas Gadjah Mada, Yogyakarta, Indonesia
Tel: +6281328850909
E-mail:

tyas_utami@ugm.ac.id

1. Introduction

Black soybean (*Glycine max* (L.) Merr.) includes high quantities of vitamin B (niacin, pantothenic acid and riboflavin) [1,2], minerals (Ca, Fe, Zn and Mn) [3,4] and proteins with diverse amino acid contents [5,6]. Black soybean is rich in bioactive compounds such as anthocyanin [7] and isoflavone [8] with antioxidant properties. Like other legumes, black soybean can be processed into soymilk. Antioxidant compounds such as isoflavone in soymilk are majorly in glucoside form [9]. Isoflavone glucoside is more water-soluble and polar. Its chemical and physical

properties produce difficulties to pass the intestinal epithelia and to absorb [10]. However, the biochemical aglycone form is absorbed faster at higher quantities [11]. Naturally, isoflavone aglycone shows higher antioxidant activities. The phenolic hydroxyl group in isoflavone aglycone acts as an electron donor, causing breakdown of the chain reaction of free radicals [12]. It has been discovered that fermentation can enhance antioxidant properties of soymilk [13-15]. Lactic acid bacteria (LAB) need carbon sources for cell development and metabolic activity during fermentation. Glucose moiety in isoflavone glucoside may be used as a carbon source. Isoflavone glucoside can be hydrolyzed into isoflavone aglycone and glucose by β-glucosidase [15,16]. Strains [9] and fermen-tation condition [16,17] contribute to the production of inducible β-glucosidase by LAB. LAB's cell growth and metabolism are dependent on their ability to utilize carbon sources and the activity of their inducible enzymes.

There is a possibility that the fermentation of black soymilk by LAB can be developed into functional food products with antioxidant properties. During processing of fermented soymilk, sucrose and skim milk are added to enhance sensory properties and increase growth and acid production. Therefore, it is critical to investigate the antioxidant activity of black soymilk supplemented with sucrose and skim milk fermented by LAB. Ability of various LAB strains to develop and increase antioxidant activity through fermentation differs [9]. *Lactobacillus* (*L.*) *plantarum* WGK 4, *Streptococcus* (*S.*) *thermophilus* Dad 11 and *L. plantarum* Dad 13 have been shown to grow well in jack bean milk [18]. the *L. plantarum* WGK 4 was isolated from red lima bean soaking water from tempe productions [18], while *S. thermophilus* Dad 11 and *L. plantarum* Dad 13 used as starters for milk fermentation were isolated from traditionally fermented buffalo milk (dadih) from West Sumatra, Indonesia [19]. Ability of these indigenous LAB strains to enhance antioxidant activity in black soymilk are still unknown. Therefore, this study investigated the antioxidant properties of black soymilk and black soymilk supplemented with 2% w v^{-1} sucrose or skim milk using selected Indonesian indigenous LAB. Moreover, the β-glucosidase activity and ability of these indigenous LAB to grow and produce acid in black soymilk and black soymilk supplemented with 2% w v^{-1} sucrose or skim milk were assessed. In general, 2% w v^{-1} sucrose or 2% w v^{-1} skim milk was selected because addition of 2-10% w v^{-1} did not significantly affect growth and acid production while 0 and 2% were significantly different [20].

2. Materials and Methods

2.1 Materials

Black soybean seed (*Glycine max* (L.) Merr.) Detam-1 variety was purchased from UPBS Balitkabi, Malang, Indonesia. The *L. plantarum* WGK 4 was provided by the Laboratory of Biotechnology, Department of Food and Agricultural Product Technology, Faculty of Agricultural Technology, Universitas Gadjah Mada. The, *S. Thermophilus* Dad 11 and *L. plantarum* Dad 13 were provided by the Food and Nutrition Culture Collection (FNCC), Center for Food and Nutrition Studies, Universitas Gadjah Mada, Yogyakarta, Indonesia. De Mann Rogosa (Merck, Germany), bacteriological agar (Oxoid, USA) and calcium carbonate ($CaCO_3$) (Merck, Germany) were used for microbial analyses. Chemical reagents such as phenolphthalein (PP), 2,2'-diphenyl-1-1-picrylhydrazyl (DPPH), Folin-Ciocalteu reagent, gallic acid, ρ-nitrophenyl-α-D-glucopyranoside (ρNPG) and daidzein and genistein standards were purchased from Sigma-Aldrich, USA. Sucrose (PT Sugar Group Company, Indonesia) and skim milk (PT Mirota KSM, Indonesia) were purchased from the local supermarkets.

2.2 Inoculum preparation [18]

Stock culture was stored in the mixture of 20% of sterile sucrose and 10% of skim milk (1:1) at -20 °C. The culture was recovered in MRS broth at 37 °C for 24 h and maintained in MRS agar media as working culture. This was stored at 4 °C and refereshed every two weeks. For the starter culture preparation, samples from working cultures were inoculated in MRS broth and incubated at 37 °C for 24 h twice. The viable cells of LAB in starter culture were assessed before inoculation into black soymilk and expressed as a colony-forming unit (CFU) ml^{-1}.

2.3 Black soymilk preparation [18]

Black soybean was washed and soaked overnight at room temperature. The soaked soybean was then drained and washed. Black soybean was blended using blender (Matsushita, Japan) with 80 °C water (1:2) for four min. Slurry was filtered twice using filter cloth and transferred into a sterile 100-ml glass bottle. Black soymilk and black soymilk with the addition of 2% (w v^{-1}) sucrose or 2% (w v^{-1}) skim milk were pasteurized at 65-70 °C for 30 min.

2.4 Fermentation of black soymilk [18]

Black soymilk and black soymilk supplemented with 2% sucrose or skim milk were inoculated with 1% of *L. plantarum* WGK 4, *S. thermophilus* Dad 11 or *L. plantarum* Dad 13. Fermentation was carried out in an incubator (Sanyo MIR-262, Japan) at 37 °C for 18 h. Viable cell, acid production (titratable acidity and pH), β-glucosidase activity, isoflavone aglycone (daidzein and genistein), total phenolic content (TPC) and antioxidant activities were assessed at the begining of the experiment and after 18 h of the fermentation of black soymilk .

2.5 Assessment of the cell growth and acid production [18]

The viable cell was assessed using serial dilution and pour plate methods with MRS media, includeing 1.5% of bacteriological agar and 0.5% of calcium carbonate ($CaCO_3$). The pH was assessed using pH meter (HANNA HI 2210, UK) and titratable acidity was analyzed using titration with 0.1 N sodium hydroxide (NaOH) and phenolphthalein (PP) as indicators.

2.6 Assessment of the β-glucosidase activity in fermented black soymilk [21]

Hydrolysis rate of the ρ-nitrophenyl-α-D-glucopyranoside (ρNPG) substrate was carried out to assess activity of the β-glucosidase. The crude enzyme was achieved by centrifugation (Thermo Fisher Scientific, USA) of 10 ml of the fermented black soymilk at 2576× g for 15 min at 4 °C. The crude enzyme was included in the the supernatant. The crude enzyme (500 µl) was added into 1 ml of 5 mM ρNPG prepared in 100 mM sodium phosphate buffer (pH 7) and incubated at 37 ºC for 30 min. To stop the reaction, 1 ml of cold sodium carbonate was added to the reaction and the absorbance was measured using UV-Vis spectrophotometer (Thermo Fisher Scientific Genesys 150, USA) at 401 nm. Under assay conditions, one unit of enzyme was defined as the quantities of enzyme releasing one mol of ρ-nitrophenol from ρ-NPG as substrate and from ρ-nitrophenol as standard per minute.

2.7 Assessment of the isoflavone aglycone content [22]

High-performance liquid chromatography (HPLC) was used to assess daidzein and genistein, the dominant isoflavone aglycone in soymilk. Samples were freeze-dried (Modulyo Model, Edwards, UK) before analysis. One gram of the samples was extracted for isoflavone using 50% methanol (1:10). Extraction was carried out using sonicator bath (Eyela Sonicator Cleaner, Singapore) for 30 min. Samples were then centrifuged at 1449 g for 15 min. Supernatants were filtered twice using no. 1 Whatman papers and 0.45-µm syringe filters (Sartorius Minisart, Germany) before injected into HPLC. Samples were analyzed using HPLC (LC-20AD Model, Shimadzu, Japan) equipped with autosampler (SIL-20A HT Model, Shimadzu, Japan), quaternary pump, PDA detector (CTO-20A Model, Shimadzu, Japan), degassing unit (DGU-20A SR Model, Shimadzu, Japan) and Sun Fire TMC reverse phased C-18 column (150 × 4.6 mm, 5 µm). The mobile phase included methanol (Solvent A) and 0.1% acetic acid in water (Solvent B). The flow rate was set isocratic at 1 ml min^{-1} with a ratio of solvent A to B of 53:47 for 15 min. Column temperature and detector wavelength were set at 30 °C and 254 nm, respectively. Quantities of daidzein and genistein were calculated based on standard curve (10, 20, 40, 60, 80 and 100 µg ml^{-1}).

2.8 Preparation of the phenolic crude extract of fermented black soymilk [23]

Extraction of the phenolic compounds in black soymilk was carried out for phenolic content and antioxidant activity assay. Two milliliters of the fermented black soymilk samples were extracted using 70% methanol (1:5) and water bath shaker (Sibata WS-240, Japan) at 120 rpm for 72 min at 30 °C, supported by maceration at 4 °C for 24 h at dark. After centrifuging the extracts at 3000 g for 15 min, supernatants were filtered using Whatman filter papers no. 42. Then, supernatants were re-extracted using a similar method. Crude extracts from the first and second extractions were mixed together and stored at -20 °C before analysis.

2.9 Total phenolic coumpound assay [23]

Crude extracts were diluted four times using distilled water. Samples were then transferred into a tube with 1 ml of Folin-Ciocalteu reagent, mixed and set for 1 min. Then, 4 ml of 15% sodium carbonate (Na2CO3) were added to the mixture. Samples were remixed using vortex and incubated at dark for 2 h at room temperature. Absorbance of the resultant blue complex was assessed at 760 nm using UV-Vis spectrophotometer. Methanol was used as blank and gallic acid as standard. Results were expressed in mg gallic acid equivalent (GAE) per 100 ml.

2.10 2,2'-diphenyl-1-picrylhydrazyl radical scavenging assay [24]

Capacity of the extracts to scavenge 2,2'-diphenyl-1-picrylhydrazyl (DPPH) radical was used to assess antioxidant activity. One milliliter of the crude extracts was transferred into a tube and mixed with 3 ml of 0.1 mM DPPH solution and incubated at ambient temperature for 30 min at dark. Absorbance was assessed at 515 nm using UV-Vis spectrophotometer. Methanol was used as control. The atioxidant activity was assessed using the following equation:

$$Scavenging\ activity\ (\%) = (1 - \frac{samples\ absorbance}{control\ absorbance}) \times 100\%$$

2.11 Statistical analysis

Results were provided as means ±SD (standard deviation). In this study, IBM SPSS v.20 (n = 4), one-way ANOVA and Duncan's multiple range test with a significance value of $p < 0.05$ were used for the statistical analysis.

3. Results and Discussion

3.1 Growth and acid production of the LAB in fermented black soymilk

without sucrose or skim milk supplementation. Table 1 shows Indonesian indigenous LAB growth and acid production in the fermented black soymilk with and without sucrose or skim milk supplementation

Table 1. Viable cell, pH and titratable acidity of the fermented black soymilk and fermented black soymilk with supplementation of 2% sucrose or skim milk using selected lactic acid bacteria (37 °C, 18 h)

Strains	Supplementation	Viable cell (log CFU ml^{-1})		pH		Titratable acidity (%)	
		Initial	Final	Initial	Final	Initial	Final
Lactobacillus	0%	7.32±0.12^{a}	9.52±0.14^{b}	6.37±0.05^{c}	4.23±0.06^{b}	0.38±0.05^{a}	1.24±0.10^{b}
plantarum	2% Sucrose	7.32±0.12^{a}	9.26±0.49^{b}	6.40±0.05^{cd}	4.05±0.02^{a}	0.38±0.01^{a}	1.35±0.10^{b}
WGK 4	2% Skim	7.32±0.12^{a}	9.45±0.31^{b}	6.46±0.03^{d}	4.19±0.01^{b}	0.43±0.09^{a}	1.30±0.12^{b}
Streptococcus	0%	7.34±0.25^{a}	9.38±0.26^{b}	6.37±0.05^{c}	4.66±0.07^{b}	0.38±0.05^{a}	0.88±0.08^{b}
thermophilus	2% Sucrose	7.34±0.25^{a}	9.34±0.17^{b}	6.40±0.05^{cd}	4.12±0.02^{a}	0.38±0.01^{a}	1.32±0.05^{c}
Dad 11	2% Skim	7.34±0.25^{a}	9.49±0.09^{b}	6.46±0.03^{d}	4.12±0.07^{a}	0.43±0.09^{a}	1.54±0.17^{d}
Lactobacillus	0%	7.60±0.05^{a}	9.49±0.13^{b}	6.37±0.05^{d}	4.67±0.04^{c}	0.38±0.05^{a}	0.92±0.11^{b}
plantarum Dad	2% Sucrose	7.60±0.05^{a}	9.56±0.26^{b}	6.40±0.05^{de}	4.14±0.03^{a}	0.38±0.01^{a}	1.22±0.14^{c}
13	2% Skim	7.60±0.05^{a}	9.52±0.13^{b}	6.46±0.03^{e}	4.24±0.02^{b}	0.43±0.09^{a}	1.35±0.20^{c}

Values are expressed as mean ±SD. Values of each strain with different superscripts are significantly different ($p < 0.05$) using Duncan's multiple range test.

The LAB count for all strains increased nearly 2 log cycles from 7.32-7.60 to 9.34-9.56 log CFU ml^{-1} after 18 h of fermentation of all black soymilk samples. There was no statistically significant difference between fermented black soymilk with and without sucrose or skim milk addition. The results indicated that all of the strains could grow well in black soymilk and black soymilk with 2% sucrose or skim milk supplementation. Lee et al. [14] reported that the *S. thermophilus* S10 count reached nearly 10^{8}-10^{9} CFU ml^{-1} in black soymilk after 24 h of fermentation. Yudianti et al. [18] revealed that the growth of *L. plantarum* WGK 4 in jack bean milk increased 1.07 log CFU ml^{-1}. Moreover, Fitrotin et al. [17] explained that sucrose addition did not significantly increase growth of *L. plantarum* Dad 13 during fermentation of sesame milk; similar to the current results. The LAB utilized nutrients in black soymilk for their growth and metabolic activities led to the production of acid. Acid production was assessed using titratable acidity and pH assays. Titratable acidity significantly increased with the decreased pH in all black soymilk samples, showing acid production during fermentation. Supplementation of 2% sucrose or skim milk increased acid production described by the higher titratable acidity and lower pH in *S. thermophilus* Dad 11 and *L. plantarum* Dad 13 but not in *L. plantarum* WGK 4. similar results were investigated by Wardani et al. [25] thattitratable acidity of the fermented milk using *L. plantarum* Dad 13 after 18 h of incubation was 0.75% and the pH was 3.99. Djaafar et al. [26] reported that titratable acidity and pH of the kerandang milk after 24 h of fermentation with *L. pentosus* T14 was nearly 0.9% and 4.60, respectively. Furthermore, Utami et al. [20] demonstrated that the supplementation with sucrose significantly increased titratable acidity and decreased pH of the peanut milk fermented with *L. paracasei* SNP-2. Similar results were achieved in the current study.

The current indigenous LAB could grow well in black soymilk with no sucrose or skim milk supplementation. This might be due to the bacterial ability to use fermentable sugars such as sucrose [23] in black soymilk. Sucrose was the predominant soluble sugar in black soybean [27]. Naturally, LAB need nitrogen sources and growth factors to grow. Moreover, *S. thermophilus* needs histidine and cysteine or methionine to grow, while *L. plantarum* needs arginine, glutamic acid, leucine, valine and cysteine or methionine [28]. The LAB need vitamin B such as niacin and pantothenic acid as growth factor [29]. Black soybean included these amino acids [5] and vitamins [1]. Hence, LAB could grow well. Furthermore, black soybean included several minerals such as Fe, Zn and Mn [4] needed by LAB for the enzymatic reactions [29]. Supplementation of 2% sucrose or skim milk increased acid production but did not affect growth of *S. thermophilus* Dad 11 and *L. plantarum* Dad 13. This indicated that nutrients in black soymilk were enough for cell growth but not for acid production. Sucrose and skim milk additions in fermentation of black soymilk by *L. plantarum* WGK 4 did not significantly increase acid production. It might be due to *L. plantarum* WGK 4 isolated from legume soaking water, which might be able to utilize sucrose and oligosaccharides for its growth and metabolic activity. In contrast, *L. plantarum* Dad 13 and *S.*

thermophilus Dad 11 isolated from fermented buffalo milk might include lower abilities to utilize oligosaccharides. Therefore, supplementation of 2% sucrose or skim milk increased acid production in fermented black soymilk by *L. plantarum* Dad 13 and *S. thermophilus* Dad 11. The current results showed that these three Indonesian indigenous LAB could grow and produce acid in black soymilk and black soymilk with 2% sucrose or skim milk supplementation.

3.2 β-glucosidase activity and isoflavone aglycone of the fermented black soymilk

To use nutrients for metabolism activity, LAB may produce inducible enzymes such as β-glucosidase. The β-glucosidase can hydrolyze isoflavone glycoside to produce isoflavone aglycone and glucose, providing fermentable sugars for LAB. The β-glucosidase activities of the indigenous LAB in fermented black soymilk and fermented black soymilk supplemented with 2% sucrose or skim milk are presented in Figure 1. All strains showed the highest activites of β-glucosidase in fermented black soymilk with no sucrose or skim milk supplementation. These included nearly 20 mU ml^{-1}. Lower β-glucosidase activities were found in fermented black soymilk supplemented with 2% sucrose or skim milk. Ability of the LAB to produce this inducible enzyme is strain-dependent [9] and affected by fermentation media [16,17]. Results were similar to those of β-glucosidase activity within 12 h of fermentation of soymilk with *Saccharomyces boulardii* combined with *L. plantarum* B4495 [30]. Titiek et al. [21] reported that the β-glucosidase activities of *L. plantarum* T33 and *L. plantarum pentosus* T35 during fermentation of kerandang extracts were respectively 20 and 18 mU ml^{-1} culture; similar to those from the present study. Fitrotin et al. [17] described that the activity of β-glucosidase in fermentation of sesame milk was 70.3 U ml and sucrose significantly decreased the β-glucosidase activity. Other studies revealed that the β-glucosidase activity of *S. thermophilus* S10 in soymilk after 18 h of fermentation was nearly 60 UA g^{-1} [14]. Various β-glucosidase activities of the present and previous studies have shown that the bacterial ability to produce β-glucosidase depends on the microbial strain and fermentation medium. These findings were supported by Hati et al. [9], who found a higher β-glucosidase activity in soymilk fermented with *L. rhamnosus* C6 than that soymilk fermented with *L. rhamnosus* C2, *L. rhamnosus* NCDC19, *L. rhamnosus* NCDC24, *L. casei* NCDC17 and *L. casei* NCDC297.

The current results demonstrated a lower β-glucosidase activity in fermented black soymilk supplemented with 2% sucrose or skim milk. It could be due to the fact that fermentable sugar in black soymilk was enough for the bacterial metabolic activity due to the addition of sucrose or skim milk into black soymilk; therefore, β-glucosidase activity was lower.

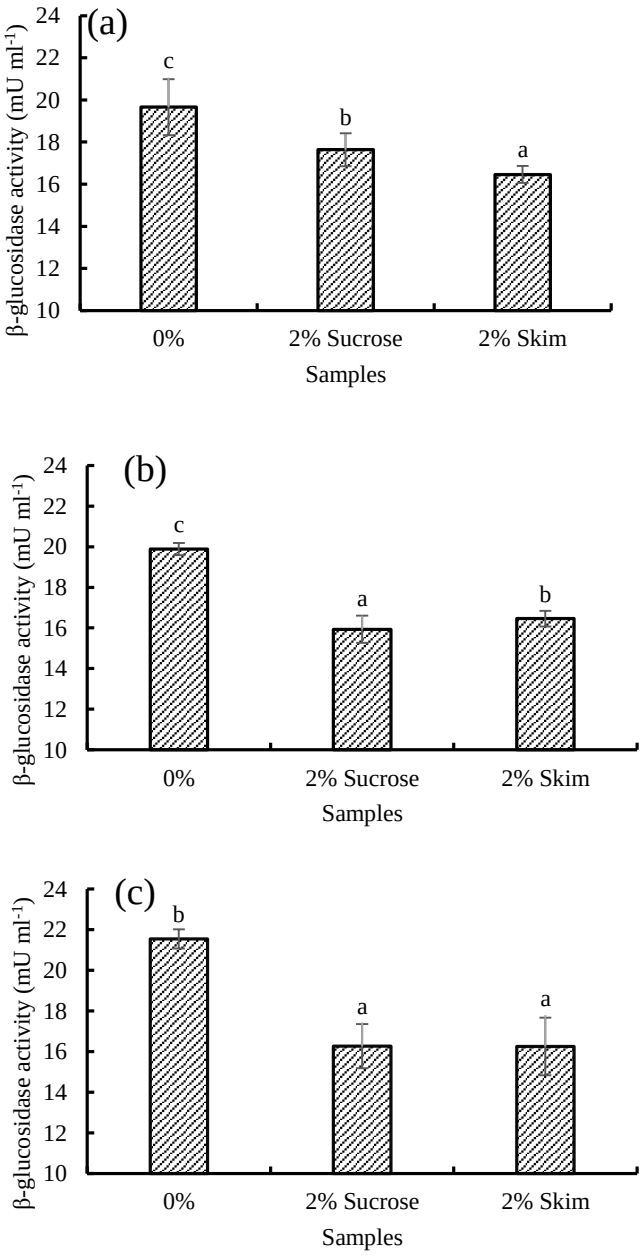

Figure 1. β-glucosidase activity of the fermented black soymilk and fermented black soymilk with supplementation of 2% sucrose or skim milk (37 °C, 18 h) by (a) *Lactobacillus plantarum* WGK 4, (b) *Streptococcus thermophilus* Dad 11 and (c) *Lactobacillus plantarum* Dad 13. Values are expressed as mean ±SD. Values of each strain with different superscripts are significantly different ($p < 0.05$) using Duncan's multiple range test

The β-glucosidase is an inducible enzyme, which is synthesized when it is needed such as when limited fermentable sugars are available. Addition of 2% sucrose or skim milk ensures carbon source adequacy and hence lower β-glucosidase activity.

In this study, activity of β-glucosidase resulted in the hydrolysis of isoflavone glucoside into glucose and isoflavone aglycone. Table 2 presents isoflavone aglycone (daidzein and genistein) concentration of the fermented

black soymilk and fermented black soymilk supplemented with 2% sucrose or skim milk. All samples showed increases in daidzein and genistein through the fermentation. Fermented black soymilk without 2% sucrose or skim milk included the highest daidzein increases of nearly 74, 72 and 62% for *L. plantarum* WGK 4, *S. thermophilus* Dad 11 and *L. plantarum* Dad 13, respectively. Genistein showed similar patterns of nearly 79, 80 and 67% for *L. plantarum* WGK 4, *S. thermophilus* Dad 11 and *L. plantarum* Dad 13, respectively. All strains produced higher isoflavone aglycone in black soymilk rather than black soymilk supplemented with 2% sucrose or skim milk. Daidzein was found in higher quantities, compared to genistein. Several studies investigated increased isoflavone aglycone after fermentation [9,16]. Liu et al. [13] reported that isoflavone glucoside daidzin, genistin and glycitin decreased as the aglycone form increased in soybean milk fermented by *L. plantarum* CQPC01. Lee et al. [14] demonstrated that daidzein and genistein in fermented black soymilk were respectively 114.14 and 186.62 mg 100 g^{-1} after 18 h using *S. thermophilus* S10. These findings were similar to those from the current study. Fitrotin et al. [17] reported that supplementation with sucrose decreased hydrolysis of sesaminol triglucoside in fermented sesame milk using *L. plantarum* Dad 13; similar to reports from the present study. In the present study, LAB produced β-glucosidase during fermentation of black soymilk to breakdown isoflavone glucoside into glucose and isoflavone aglycone and thus increased concentrations of daidzein and genistein [14]. Supplementation of 2% sucrose or skim milk in black soymilk provided an additional carbon source for the growth of LAB and their metabolic activities. Therefore, hydrolysis of isoflavone glucoside in fermented black soymilk was higher than that in black soymilk with 2% sucrose or skim milk. Moreover, *L. plantarum* WGK 4, *S. thermophilus* Dad 11 and *L. plantarum* Dad 13 produced higher β-glucosidase quantities in black soymilk with no sucrose or skim milk addition, resulting in higher daidzein and genistein quantities. Release of isoflavone aglycone might affect TPC and antioxidant capacity of the black soymilk.

3.3 Total phenolic content and antioxidant activity of the fermented black soymilk

Increased isoflavone aglycone by the β-glucosidase activity during fermentation could enhance TPC and antioxidant activity of the black soymilk. The TPC in fermented black soymilk with and without supplementation of sucrose or skim milk is shown in Figure 2.

Supplementation of skim milk in unfermented black soymilk increased its TPC. All strains showed increases in TPC after fermentation of black soymilk from 31.44 to 44.44, 35.94 and 37.66 mg GAE 100 ml^{-1} for *L. plantarum* WGK 4, *S. thermophilus* Dad 11 and *L. plantarum* Dad 13, respectively. Furthermore, TPC in fermented black soymilk with 2% sucrose or skim milk by *L. plantarum* WGK 4 was relatively stable. Relatively, decreases of TPC in fermented black soymilk by *S. thermophilus* Dad 11 and *L. plantarum* Dad 13 were reported, which was supplemented with skim milk.

Table 2. Isoflavone aglycone (daidzein and genistein) of the fermented black soymilk and fermented black soymilk with supplementation of 2% sucrose or skim milk using selected lactic acid bacteria (37 °C, 18 h)

Strain	Supplementation	Daidzein (μg g^{-1})		Genistein (μg g^{-1})	
		Initial	Final	Initial	Final
Lactobacillus plantarum WGK 4	0%	96.99±8.07^{a}	168.98±4.45^{c}	61.39±0.04^{a}	109.89±2.50^{d}
	2% Sucrose	92.09±0.45^{a}	136.05±0.42^{b}	61.02±0.45^{a}	84.43±0.32^{b}
	2% Skim	91.92±6.04^{a}	134.35±0.64^{b}	59.84±0.10^{a}	90.93±0.11^{c}
Streptococcus thermophilus Dad 11	0%	96.99±8.07^{a}	167.23±6.23^{c}	61.39±0.04^{a}	110.61±4.08^{c}
	2% Sucrose	92.09±0.45^{a}	138.95±0.52^{b}	61.02±0.45^{a}	93.26±0.11^{b}
	2% Skim	91.92±6.04^{a}	142.24±0.76^{b}	59.84±0.10^{a}	95.28±0.01^{b}
Lactobacillus plantarum Dad 13	0%	96.99±8.07^{a}	157.42±1.19^{c}	61.39±0.04^{a}	103.07±3.25^{d}
	2% Sucrose	92.09±0.45^{a}	138.18±0.84^{b}	61.02±0.45^{a}	86.76±0.15^{c}
	2% Skim	91.92±6.04^{a}	132.79±0.87^{b}	59.84±0.10^{a}	89.94±0.19^{b}

Values are expressed as mean ±SD. Values of each strain with different superscripts are significantly different ($p < 0.05$) using Duncan's multiple range test.

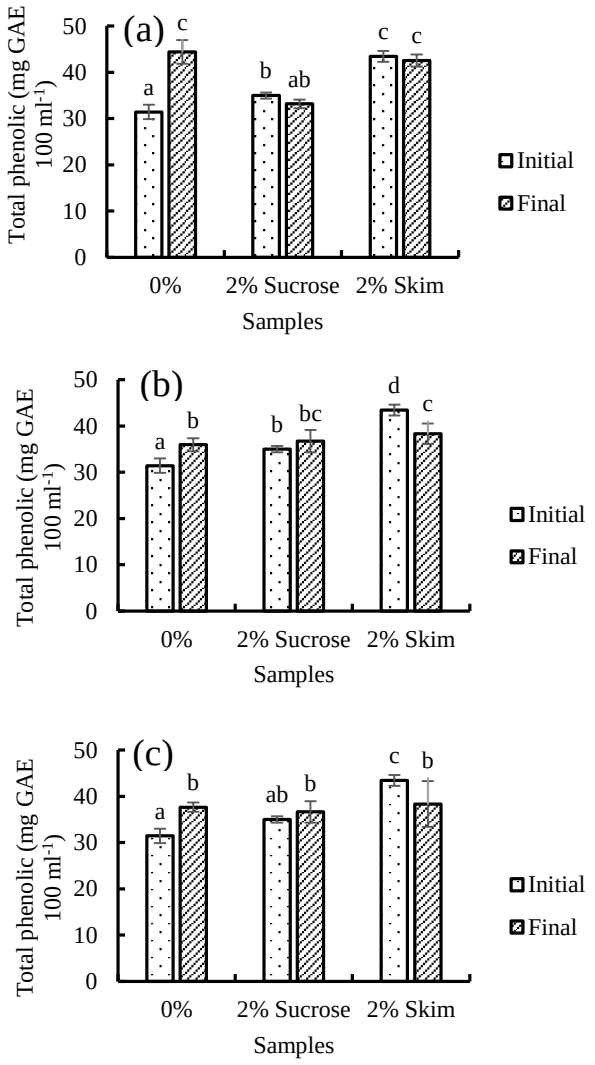

Figure 2. Total phenolic content of the fermented black soymilk and fermented black soymilk with supplementation of 2% sucrose or skim milk (37 °C, 18 h) by (a) *Lactobacillus plantarum* WGK 4, (b) *Streptococcus thermophilus* Dad 11 and (c) *Lactobacillus plantarum* Dad 13. Values are expressed as mean ±SD. Values of each strain with different superscripts are significantly different ($p < 0.05$) using Duncan's multiple range test

Fitrotin et al. [17] reported that fermented sesame milk by *L. plantarum* Dad 13 included increased TPC after 18 h of fermentation. They found that addition of sucrose significantly decreased TPC in fermented sesame milk, compared to sesame milk with no sucrose addition. These findings were similar to those from the present study. Similarly, Lee et al. [14] showed increases in TPC during black soymilk fermentation by *S. thermophilus* S10. Through the fermentation, LAB produced β-glucosidase, an enzyme responsible for catalyzing hydrolysis of β-glucosidic bonds in isoflavone glucosidic and releasing isoflavone aglycone [17]. The β-glucosidase might hydrolyze phenolic phucosides to release free phenolics [31], resulting in a higher TPC. Release of complex phenolic compounds could increase TPC. The phenolic compound interacted with polysaccharides in cell walls and proteins [32]. Polyphenol bound proline-rich regions in soybean glycinin and β-conglycinin [33]. Fermentation induced breakdown of carbohydrate or protein phenolic complex, releasing these bound phenolic compounds [34]. Meanwhile, 2% sucrose and skim milk supplementation increased carbon source availability and hence did not induce release of complex phenolics. Black soymilk supplemented with skim milk presented a higher TPC because skim milk included phenolic compounds [35]. Decreased TPC after fermentation of skim milk supplemented black soymilk by *S. thermophilus* Dad 11 and *L. plantarum* Dad 13 might occur due to the hydrolysis of phenolic compounds. Adebo et. al [34] reported that LAB could degrade phenolic compounds during fermentation. Higher phenolic compounds inhibit LAB growth [36]. Sometime, LAB produce decarboxylase and reductase to hydrolyze those phenolic compounds as a stress response [37]; therefore, decreased TPC was partially reported in strains after fermentation of black soymilk supplemented with skim milk.

Antioxidant activities of the fermented black soymilk and fermented black soymilk supplemented with sucrose or skim milk are shown in Figure 3 using DPPH radical scavenging activity. Significant increases of 22.28-26.73 to 32.01-38.77% were reported in DPPH scavenging activity after fermentation by the three bacterial strains. All fermented black soymilks showed similar antioxidant activities of 10 ppm ascorbic acid.

The *L. plantarum* WGK 4 fermentation of black soymilk with no supplementation of 2% sucrose or skim milk included a higher antioxidant activity, compared to that other bacterial fermentations did. No significant differences were seen between the antioxidant activities of *S. thermophilus* Dad 11 and *L. plantarum* Dad 13 in fermented black soymilks with or without sucrose or skim milk supplementation. Results from the present study were similar to those from the previous studies. Liu et al. [13] investigated that soybean milk fermented with *L. bulgaricus* included nearly 30% DPPH scavenging activity. Ulyatu et al. [23] reported that DPPH scavenging activity of the sesame milk fermented with *L. plantarum* Dad 13 was 39.59% after 12 h of fermentation. Fitrotin et al. [17] reported that sucrose supplementation decreased antioxidant capacity of the fermented sesame milk. The current study demonstrated increases in antioxidant activity following increased TPC. However, the antioxidant activity increased even when the TPC did not change. Naturally, fermentation by LAB released antioxidative components initially presented in inactive forms such as isoflavone through β-glucosidase activity [38]. The free isoflavone aglycone was further reactive in scavenging radicals, resulting in a greater antioxidant activity.

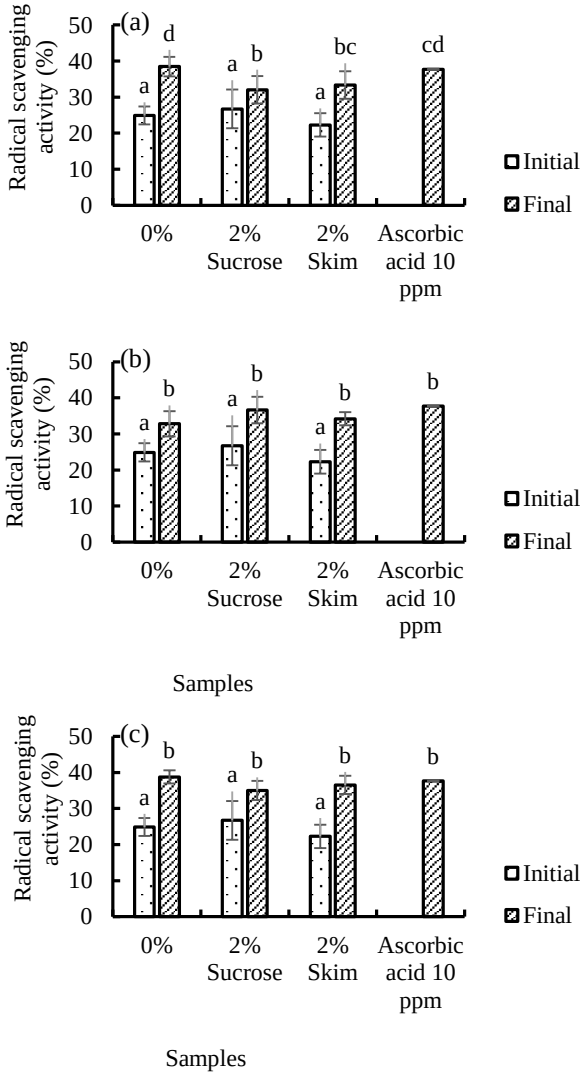

Figure 3. DPPH scavenging activity of the fermented black soymilk and fermented black soymilk with supplementation of 2% sucrose or skim milk (37 °C, 18 h) by (a) *Lactobacillus plantarum* WGK 4, (b) *Streptococcus thermophilus* Dad 11 and (c) *L. plantarum* Dad 13. Ascorbic acid was used as reference. Values are expressed as mean ±SD. Values of each strain with different superscripts are significantly different ($p < 0.05$) using Duncan's multiple range test

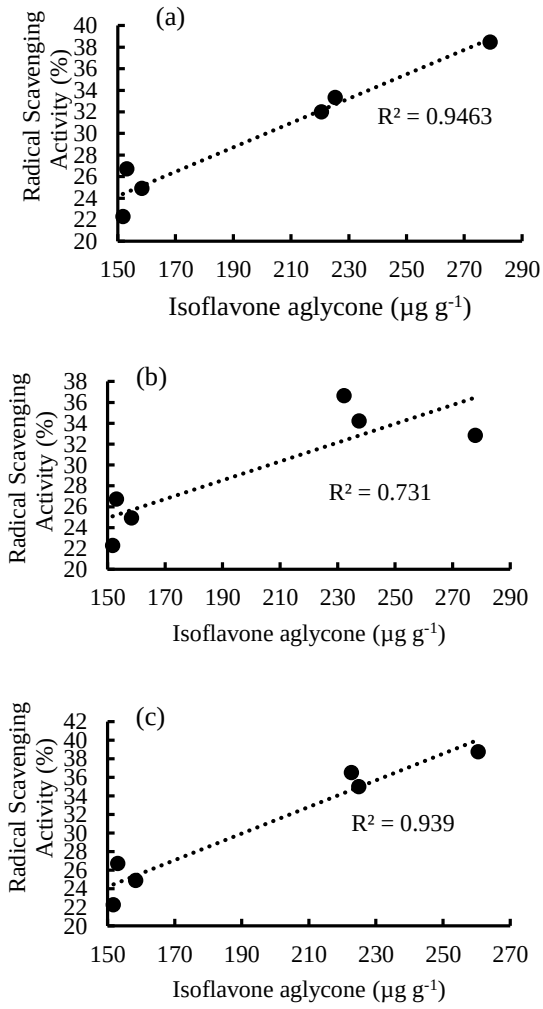

Figure 4. Relationships between isoflavone aglycone content and DPPH scavenging activity of the fermented black soymilk and fermented black soymilk with supplementation of 2% sucrose or skim milk by (a) *Lactobacillus plantarum* WGK 4, (b) *Streptococcus thermophilus* Dad 11 and (c) *Lactobacillus plantarum* Dad 13

Meanwhile, isoflavone glucoside (daidzin, genistin and dan glycitin) was not linked to antioxidant activity in black soybean [39]. Figure 4 shows relationships between isoflavone aglycone concentration and antioxidant activity of the fermented black soymilk.

The *L. plantarum* WGK 4 and *L. plantarum* Dad 13 demonstrated good correlations between the isoflavone aglycone concentration and the antioxidant activity. In contrast, *S. thermophilus* Dad 11 showed a poor correlation between these two parameters. Supplementation with 2% sucrose and skim milk significantly decreased antioxidant activity of the fermented black soymilk by *L. plantarum* WGK 4. However, this was not seen using other bacterial strains. This might be due to the release of other antioxidant compounds in black soymilk, including anthocyanins [7] or bioactive peptides [40]. Therefore, supplementation with sucrose or skim milk could be carried out at the end of the fermentation process for the production of fermented black soymilk products with high isoflavone aglycone contents.

4. Conclusion

In conclusion, *L. plantarum* WGK 4, *S. thermophilus* Dad 11 and *L. plantarum* Dad 13 could grow well in black soymilk. Supplementation with 2% sucrose or skim milk did not affect growth of the strains but increased acid production by *S. thermophilus* Dad 11 and *L. plantarum* Dad 13 during fermentation of black soymilk. Moreover, supplementation with 2% sucrose or skim milk decreased β-glucosidase activity, resulting in lower isoflavone aglycone in all strains. The present study showed that Indonesian indigenous LAB could enhance the antioxidant activity by releasing isoflavone aglycone (daidzein and genistein) in the presence of appropriate substrares and include great potentials for the development of functional foods. Further studies are necessary to investigate formation of other antioxidant compounds such as bioactive peptides during the fermentation of black soymilk by these Indonesia indigenous LAB.

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6. Conflict of Interest

The authors report no conflicts of interest.

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فعالیت ضداکسایشی ایزوفلاون آگیلاکون به‌دست آمده از شیرسویای سیاه تخمیری حاوی مکمل ساکارز وشیر بدون چربی با استفاده از باکتری‌های لاکتیک اسید بومی اندونزی

بندیکتوس یودا لکسونو[1]، محمد نور کاهیانتو[1]، تیاس اوتامی[1و2*]

۱- گروه فناوری فرآورده‌های کشاورزی و مواد غذایی، دانشکده فناوری کشاورزی، دانشگاه گدجه مادا ، یوگیاکارتا ، اندونزی
۲- مرکز مطالعات تغذیه و مواد غذایی، دانشگاه گدجه مادا ، یوگیاکارتا ، اندونزی

چکیده

سابقه و هدف: رقم Detam-1 سویای سیاه (*Glycine max* (L.) Merr.) حاوی مقادیر زیادی ایزوفلاون، عمدتا به شکل گلوکوزید می‌باشد؛ که فعالیت ضداکسایشی بیوشیمیایی کمتری از شکل ایزوفلاون آگلیکون دارد. تخمیر توسط باکتری‌های لاکتیک اسید می‌تواند فعالیت ضداکسایشی و ایزوفلاون آگلیکون شیر سویای سیاه را افزایش دهد. اگرچه، توانایی بیوشیمیایی وابسته به سوش است و مکمل ساکارز یا شیر بدون چربی در حین فرایند بر این توانایی می‌تواند اثر داشته باشد. هدف مطالعه حاضر بررسی خواص ضداکسایشی شیرسویای سیاه تخمیرشده حاوی ۲ درصد ساکارز یا شیر بدون چربی با استفاده از سه باکتری لاکتیک اسید بومی اندونزی، به نام‌های *لاکتوباسیلوس پلانتاروم* WGK 4، *استرپتوکوکوس ترموفیلوس* Dad 11 و *لاکتوباسیلوس پلانتاروم* Dad 13 بود. علاوه بر این رشد سلولی و تولید اسید مورد بررسی قرار گرفت.

مواد و روش ها: تخمیر شیرسویای سیاه و شیرسویای سیاه حاوی مکمل به میزان ۲ درصد ساکارز یا شیر بدون چربی با استفاده از سه باکتری لاکتیک اسید بومی اندونزی در ۳۷ درجه سلسیوس و به‌مدت ۱۸ ساعت انجام شد. سلول زنده، pH، اسیدیته قابل تیتراسیون، فعالیت بتا-گلوکوزیداز، ایزوفلاون آگلیکون، میزان کل ترکیبات فنولی و فعالیت ضداکسایشی شیرسویای سیاه از آغاز آزمون و پس از ۱۸ ساعت تخمیر بررسی شدند.

یافته‌ها و نتیجه‌گیری: نتایج نشان داد که تمام سویه‌ها توانایی رشد (۹log CFU ml^{-1}) و تولید اسید در شیرسویای سیاه و شیرسویای سیاه حاوی دو درصد ساکارز یا شیر بدون چربی را دارند. تخمیر از طریق فعالیت بتا-گلوکوزیداز، میزان ایزوفلاون آگلیکون را افزایش داد، که نتیجه اش افزایش میزان کل ترکیبات فنولی و فعالیت ضداکسایشی بود. شیرسویای سیاه فاقد ساکارز یا شیر بدون چربی بالاترین فعالیت بتا-گلوکوزیداز (۱۹/۲۱-۶۶/۵۴mU ml^{-1})، تشکیل دایدزین (۶۲-۷۴٪)، تشکیل جنیستئین (۶۷-۸۰٪) و ظرفیت ضداکسایشی (۳۲/۳۸-۸۱/۴۷-) را نشان داد. هر سه سوش باکتری لاکتیک اسید موجب افزایش فعالیت ضداکسایشی و ایزوفلاون آگلیکون شیرسویای سیاه شدند. ساکارز یا شیر بدون چربی اثری بر رشد سلولی نداشتند، اما تولید اسید را افزایش و فعالیت بتا-گلوکوزیداز و تشکیل ایزوفلاون آگلیکون را کاهش دادند. این سه باکتری لاکتیک اسید توانایی یکسانی برای افزایش فعالیت ضداکسایشی و تشکیل ایزفلاون آگلیکون در شیرسویای سیاه تخمیرشده داشتند.

تعارض منافع: نویسندگان اعلام می‌کنند که هیچ نوع تعارض منافعی مرتبط با انتشار این مقاله ندارند.

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واژگان کلیدی
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- جنیستئین
- *لاکتوباسیلوس پلانتاروم*
- *استرپتوکوکوس ترموفیلوس*

***نویسنده مسئول**
تیاس اوتامی
گروه فناوری فرآورده‌های کشاورزی و مواد غذایی، دانشکده فناوری کشاورزی، دانشگاه گدجه مادا ، یوگیاکارتا ، اندونزی، مرکز مطالعات تغذیه و مواد غذایی، دانشگاه گدجه مادا ، یوگیاکارتا ، اندونزی
تلفن: ۰۹۰۹ ۸۸۵۰ ۳۲۸ ۸۱۳ ۶۲+
دورنگار: ۶۲۷۴۵۸۹۷۹۷+
پست الکترونیک:
tyas_utami@ugm.ac.id