*fermentation*

MDPI

*Article*

# Enhancement of Antioxidant Activities in Black Soy Milk through Isoflavone Aglycone Production during Indigenous Lactic Acid Bacteria Fermentation

**Benediktus Yudo Leksono [1,2], Muhammad Nur Cahyanto [1], Endang Sutriswati Rahayu [1,3], Rini Yanti [1] and Tyas Utami [1,*]**

[1] Department of Food and Agricultural Product Technology, Faculty of Agricultural Technology, Universitas Gadjah Mada, Flora Street, Bulaksumur, Yogyakarta 55281, Indonesia; benediktus.leksono@uajy.ac.id (B.Y.L.); mn_cahyanto@ugm.ac.id (M.N.C.); endangsrahayu@ugm.ac.id (E.S.R.); riniyanti@ugm.ac.id (R.Y.)
[2] Faculty of Biotechnology, Universitas Atma Jaya Yogyakarta, Yogyakarta 55281, Indonesia
[3] Center for Food and Nutrition Studies, Universitas Gadjah Mada, Yogyakarta 55281, Indonesia
* Correspondence: tyas_utami@ugm.ac.id

**Abstract:** Black soybeans contain high antioxidant compounds such as isoflavone but mainly in glucoside form, with low antioxidant activities. Fermentation by lactic acid bacteria (LAB) can enhance the antioxidant properties, but its ability is strain-dependent. This study aims to study the ability of Indonesian indigenous LAB, *Lactiplantibacillus plantarum* WGK 4, *Streptococcus thermophilus* Dad 11, and *Lactiplantibacillus plantarum* Dad 13, to enhance the antioxidant properties during black soy milk fermentation. Fermentation was carried out at 37 °C for 24 h. Viable cell, acid production, Folin–Ciocalteu assay, antioxidant activity (DPPH), isoflavone aglycone daidzein and genistein, and β-glucosidase activity were measured every six hours. All LAB strains could grow well during the fermentation of black soy milk. *Lactiplantibacillus plantarum* WGK 4 produced the highest acid (1.50%). All three LAB strains could enhance antioxidant activity (DPPH) from 24.90% to 31.22–38.20%, followed by increased isoflavone aglycone. All strains could increase daidzein and genistein content, ranging from 61% to 107% and 81% to 132%, respectively. All three Indonesian indigenous LAB enhanced antioxidant properties of black soy milk relatively at the same level and potentially could be used as a starter culture of black soy milk fermentation.

**Keywords:** antioxidant activity; black soy milk; daidzein; fermentation; genistein; lactic acid bacteria



**Citation:** Leksono, B.Y.; Cahyanto, M.N.; Rahayu, E.S.; Yanti, R.; Utami, T. Enhancement of Antioxidant Activities in Black Soy Milk through Isoflavone Aglycone Production during Indigenous Lactic Acid Bacteria Fermentation. *Fermentation* **2022**, *8*, 326. https://doi.org/10.3390/fermentation8070326

Academic Editors: Michela Verni and Carlo Giuseppe Rizzello

Received: 15 June 2022
Accepted: 8 July 2022
Published: 13 July 2022



## 1. Introduction

Black soybeans (*Glycine max* (L.) Merr.) provide health benefits due to their high protein, fiber, vitamin B, and mineral content [1–4]. Black soybeans also contain many bioactive compounds, such as anthocyanin and isoflavones, which can act as antioxidants [5,6]. This points out the potential of black soybean to be developed into various functional food products. Unfortunately, the utilization of black soybeans is still not diverse.

Like yellow soybean, black soybean could be processed into soy milk. Nevertheless, isoflavones in soy milk are mostly in glucoside form [7]. In glucoside form, isoflavones are attached to glucose. Isoflavone glucosides are more water-soluble and polar compared to their aglycone form. Thus, it is difficult to pass intestinal epithelium and be absorbed [8]. Moreover, isoflavone aglycone, without glucose moiety, exhibits higher antioxidant activity. Its hydroxyl group can react with free radicals, act as an oxygen donor, and terminate the chain reaction of free radicals [9].

Several studies have proven that soy milk fermentation by lactic acid bacteria (LAB) can enhance antioxidant activities by releasing isoflavone aglycone. Lactic acid bacteria need carbon sources for cell growth and their metabolic activity. Glucose moiety

in isoflavone glucoside might be used as a carbon source. Isoflavone glucoside can be hydrolyzed by β-glucosidase into glucose and isoflavone aglycone, thus increasing the antioxidant activities [10]. Zhao and Shah [11] reported that fermentation by several LAB increased the antioxidant level of soy milk due to deglycosylation of isoflavones glucoside. In addition, Lee et al. [12] stated that *Streptococcus thermophilus* S10 produced β-glucosidase during fermentation and hydrolyzed isoflavone glucoside into its aglycone form, increasing the antioxidant activities of soy milk. Furthermore, Hati et al. [7] studied the ability of six different strains of *Lactobacillus rhamnosus* and *Lactobacillus casei* to produce β-glucosidase and bioconvert isoflavone glucoside into isoflavone aglycone during fermentation and found out that each strain produced significantly different bioconversion of the glucoside isoflavones. This points out the potential of black soybean to be developed into fermented non-dairy milk that possesses functional properties. Nevertheless, these findings indicated that the ability of LAB to enhance antioxidant activity by producing β-glucosidase to hydrolyze isoflavone glucoside is strain-dependent.

Even though previous research already reported that some LAB strains could enhance antioxidant activity, their ability differs among strains [7] and fermentation conditions [10,13]. *Lactiplantibacillus plantarum* WGK 4, *Streptococcus thermophilus* Dad 11, and *Lactiplantibacillus plantarum* Dad 13 are some indigenous LAB strains that have been isolated in previous studies from various sources. *Lactiplantibacillus plantarum* WGK 4 was isolated from red lima bean soaking water in tempeh production [14], while *Streptococcus thermophilus* Dad 11 and *Lactiplantibacillus plantarum* Dad 13 was isolated from dadih, Indonesian traditional fermented buffalo milk [15]. These LAB could grow well in milk and jack bean milk [14–16]. The ability of these three indigenous LAB to grow and enhance their antioxidant properties by increasing the isoflavone aglycone content during the fermentation of black soy milk is still unknown. Additionally, most of the previous research studied the fermentation of yellow soy milk. Studies on the fermentation of black soy milk are still limited. Black soybeans have a different nutritional composition and might affect the growth of LAB. Moreover, previous studies have not investigated sugar utilization during fermentation, whereas simple sugar availability might affect isoflavone degradation. Therefore, this study aims to investigate the ability of three Indonesian indigenous LAB strains, *L. plantarum* WGK 4, *S. thermophilus* Dad 11, and *L. plantarum* Dad 13, to grow and enhance antioxidant properties during the fermentation of black soy milk.

## 2. Materials and Methods

### *2.1. Materials*

*Lactiplantibacillus plantarum* WGK 4 (previously identified as *Lactobacillus plantarum* WGK 4) culture was obtained from the Laboratory of Biotechnology, Department of Food and Agricultural Product Technology, Faculty of Agricultural Technology, Universitas Gadjah Mada, Yogyakarta, Indonesia. Meanwhile, *Streptococcus thermophilus* Dad 11 and *Lactiplantibacillus plantarum* Dad 13 (previously identified as *Lactobacillus plantarum* Dad 13) cultures were obtained from Food and Nutrition Culture Collection (FNCC), Center for Food and Nutrition Studies, Universitas Gadjah Mada, Yogyakarta, Indonesia. Black soybean seed Detam-1 variety was purchased from UPBS Balitkabi, Malang Regency, Indonesia. De Mann, Rogosa, and Sharpe (MRS) medium was purchased from Merck (Darmstadt, Germany), and all the chemical reagent was purchased from Sigma-Aldrich (Burlington, MA, USA).

### *2.2. Inoculum Preparation*

The inoculum preparation was performed according to Yudianti et al. [15]. The stock culture was maintained in a sterile sucrose (20% $w \cdot v^{-1}$) and skim milk (10% $w \cdot v^{-1}$) mixture (1:1) at −20 °C. For working culture, the culture was incubated in MRS broth at 37 °C for 24 h and maintained in MRS deep tube agar, stored at 4 °C. Culture from working culture was activated in MRS broth and incubated at 37 °C for 24 h twice as a starter culture. The

viable cell of LAB in starter culture was measured before inoculation into black soy milk and expressed as a colony-forming unit CFU·mL$^{-1}$.

### *2.3. Preparation of Black Soy Milk and Fermented Black Soy Milk*

The black soy milk and fermented black soy milk preparation were conducted according to Yudianti et al. [15]. Black soybeans were washed and soaked overnight at room temperature before being blended in a blender (Matsushita, Japan) with water (80 °C, 1:2) for four minutes. The slurry was then filtered twice by a filter cloth, placed in a sterile 100 mL glass bottle, pasteurized at 65–70 °C for 30 min, and let cool before inoculating with LAB. Black soy milk was immediately analyzed for chemical properties such as proximate compounds, Folin–Ciocalteu assay, and antioxidant activities. Meanwhile, for isoflavone aglycone (daidzein and genistein) concentration, sugar and amino acid profile, and minerals (iron (Fe), zinc (Zn), magnesium (Mg), and manganese (Mn)) content, the sample was freeze-dried (Modulyo, Edwards, UK) and stored in a freezer (−20 °C) until further analysis. The fermentation of black soy milk was conducted by inoculating the black soy milk with a single culture of either *L. plantarum* WGK 4, *S. thermophilus* Dad 11, or *L. plantarum* Dad 13 (1% v·v$^{-1}$). Fermentation was conducted in an incubator (Sanyo MIR-262, Osaka, Japan) at 37 °C for 24 h. Fermented black soy milk was analyzed every six hours. Viable cell, titratable acidity, pH, Folin–Ciocalteu assay, antioxidant activity (DPPH), and β-glucosidase activity were immediately analyzed. Meanwhile, for the lactic and acetic acid concentration, sugar profile, and isoflavone aglycone concentration, fermented black soy milk was freeze-dried and stored in a freezer (−20 °C) until further analysis.

### *2.4. Proximate Composition Analysis*

The proximate composition of black soy milk was analyzed according to AOAC [17]. The total fat of black soy milk was determined by the Mojonnier method and the crude protein by the Kjeldahl method according to AOAC 989.05 (Fat in Milk—Modified Mojonnier Ether Extract) and 991.20 (Nitrogen (Total) in Milk—Kjeldahl Methods), respectively. Moreover, black soy milk's water and ash content was established gravimetrically according to AOAC 925.23 (Solids (total) in Milk) and 945.46 (Ash of Milk—Gravimetric Method), respectively. Meanwhile, the total carbohydrates were quantified by calculating the percentage remaining after all the other components were measured (by difference).

### *2.5. Mineral Element Analysis*

Mineral contents (Fe, Zn, Mg, and Mn) in black soy milk samples were carried out using Inductively Coupled Plasma-Optical Emission Spectrometry (Agilent Technologies 700 Series ICP-OES, Santa Clara, CA, USA) described by AOAC 2011.14 (calcium, copper, iron, magnesium, manganese, potassium, phosphorus, sodium, and zinc in Fortified Food Products. Microwave Digestion and Inductively Coupled Plasma-Optical Emission Spectrometry) [17]. Approximately 0.5 g of freeze-dried black soy milk was weighed into a vessel and digested using a 10 mL concentrated nitric acid ($HNO_3$). Digestion was conducted using microwave digestion under the following conditions: temperature—150 °C; ramp time—10 min; hold time—15 min. The digested sample was cooled down and transferred into a 50 mL volumetric flask. As an internal standard, 100 mg·L$^{-1}$ of yttrium (Y) was added to the digested sample and diluted using double distilled water till the mark. The solution was filtered using filter paper before being analyzed. Inductively coupled plasma optical emission spectrometry was performed using concentric glass as nebulizer type, the intensity of torch alignment >1,000,000 for horizontal and vertical, and the position of torch alignment −1 to 1 for horizontal and vertical. The absorbance of Fe, Zn, Mg, Mn, and Y was measured at 238 nm, 213 nm, 285 nm, 257 nm, and 371 nm, respectively. The concentration of each mineral was measured using a calibration curve of its respective standard solution.

### 2.6. Determination of Amino Acids Profile

The amino acid determination was measured using Acquity Ultra Performance Liquid Chromatography (UPLC) H-Class Amino Acid Analysis from Waters (Queenstown, Singapore) according to the kit's procedure (AccQ-Tag Ultra Chemistry Kit number 176001235). One gram of freeze-dried black soy milk was hydrolyzed using hydrochloric acid (6 N) solution. The hydrolyzed sample was transferred into a 50 mL volumetric flask and diluted using double distilled water till the mark. The diluted sample was filtered using a 0.2 µm syringe filter (Sartorius Minisart, Germany) and added with a 2.5 mM internal standard. Alpha-Aminobutyric acid (AABA) was used as an internal standard. The protein hydrolysate was derivatized using AccQ Tag Ultra reagent containing borate buffer, 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC), and acetonitrile as reagent diluent based on the procedure listed on the kit. The derivatized solution was analyzed using Acquity UPLC H-Class (Waters) system equipped with Quaternary Solvent Manager (QSM), Sample Manager—Flow Waters Corporation (SM-FTN), Column heater module (CH-A), ACQUITY UPLC BEH C18 (2.1 × 100 mm, i.d., 1.7 µm) column and ACQUITY UPLC photodiode array detector (PDA), from Waters. The mobile phase is composed of 100% AccQ Tag Ultra eluent A concentrate (A), 90:10 water: AccQ Tag Ultra eluent B (B), 100% HPLC-grade water (C), and 100% AccQ Tag Ultra eluent B (D). The samples were injected into the UPLC system under the following conditions: column and sample temperatures were 49 °C and 20 °C, respectively, the injection volume was 1 µL, pressure around 13,000 psi, and the amino acids were detected at 260 nm. The mobile phase flow rate and gradient system are shown in Table 1. The quantification of individual amino acids was based on the ratio of samples or amino acid standard area and internal standard area.

**Table 1.** Mobile phase composition for amino acids determination.

| Time (min) | Flow Rate ($mL \cdot mL^{-1}$) | % Mobile Phase A | % Mobile Phase B | % Mobile Phase C | % Mobile Phase D |
|---|---|---|---|---|---|
| Initial | 0.700 | 10.0 | 0.0 | 90.0 | 0.0 |
| 0.29 | 0.700 | 9.9 | 0.0 | 90.1 | 0.0 |
| 5.49 | 0.700 | 9.0 | 80.0 | 11.0 | 0.0 |
| 7.10 | 0.700 | 8.0 | 15.6 | 57.9 | 18.5 |
| 7.30 | 0.700 | 8.0 | 15.6 | 57.9 | 18.5 |
| 7.69 | 0.700 | 7.8 | 0.0 | 70.9 | 21.3 |
| 7.99 | 0.700 | 4.0 | 0.0 | 36.3 | 59.7 |
| 5.89 | 0.700 | 4.0 | 0.0 | 36.3 | 59.7 |
| 8.68 | 0.700 | 10.0 | 0.0 | 90.0 | 0.0 |
| 10.20 | 0.700 | 10.0 | 0.0 | 90.0 | 0.0 |

### 2.7. Determination of Sugars Profile

The sugar compounds were determined using High-Performance Liquid Chromatography (Waters, Singapore) connected to Waters 2414 Refractive Index (RI) Detector according to AOAC 980.13 (Fructose, Glucose, Lactose, Maltose, and Sucrose in Milk Chocolate—Liquid Chromatographic Method) [17]. A freeze-dried black soy milk sample (0.5 g) was weighed into a 25 mL volumetric flask and diluted with double distilled water to half the mark. The solution was sonicated (Eyela Sonicator Cleaner, Singapore) for 15 min, and 1 mL of carrez I and II solution were added and shaken until homogeneous. Double distilled water was added to the mark and shaken until homogeneous. The solution was transferred into a 2 mL tube and centrifuged (Thermo Fisher Scientific, Waltham, MA, USA) at 14,000 rpm for 3 min. The samples were filtered using GHP/RC 0.45 µm syringe tube into a 2 mL vial before being injected into the HPLC system. The chromatographic separation of sugars was achieved on a Carbohydrate column (5 µm, 250 × 4.6 mm). The injection volume was 10 µL, and the mobile phase was 80% acetonitrile at a flow rate of 1 $mL \cdot min^{-1}$ under isocratic conditions with ambient temperature. Sugar quantification was carried out using the calibration curve of an external mixed standard solution.

### *2.8. Determination of Lactic and Acetic Acid Content*

Lactic and acetic acid in black soy milk was measured based on the procedure described by [18] using Acquity Ultra Performance Liquid Chromatography (UPLC) equipped with ACQUITY UPLC photodiode array detector (PDA) from Waters (Singapore). First, 1 gram of freeze-dried black soy milk samples was weighed into a 25 mL volumetric flask, and 5 milliliters of 20 mM $H_3PO_4$ buffer solution were added. The mixture was vortexed and sonicated for 10 min at room temperature. Thereafter, 20 mM $H_3PO_4$ buffer was added to the mark and shaken until homogeneous. The solution was transferred into a 15 mL conical centrifuge tube and centrifuged at 4000 rpm for 10 min. The supernatant was cleaned up using SPE-C18. The SPE-C18 was conditioned by eluting 1.5 mL methanol and equilibrated by eluting 1 mL of 20 mM $H_3PO_4$ buffer twice before being used. A total of 1 milliliter of samples was loaded into the SPE C-18, and the eluate was discarded. The process was repeated twice using 2.5 mL samples, and the eluate was collected. Samples were filtered with a 0.2 µm syringe filter into a 2 mL vial before being injected into the UPLC-PDA system. The conditions and parameters of the instrument were as follows: stationary phase—C18 column (100 × 3.0 mm); mobile phase—20 mM $H_3PO_4$; flow rate—0.425 mL·min$^{-1}$ under isocratic conditions; run time—10 min; injection volume 10 µL; needle wash—10% acetonitrile. Lactic and acetic acid quantification was determined using an external standard calibration curve.

### *2.9. Viable Cell, Titratable Acidity, and pH Assay*

The viable cell, titratable acidity, and pH were determined by the method described by Yudianti et al. [15]. The viable cell of LAB was determined by serial dilution and pour plate method using MRS media containing 0.5% calcium carbonate ($CaCO_3$) and 1.5% bacteriological agar. Titratable acidity was measured by titrating 5 mL samples with 0.1 N sodium hydroxide (NaOH) using phenolphthalein (PP) as the indicator. Meanwhile, pH was examined using a pH meter (HANNA HI 2210, UK).

### *2.10. Preparation of Crude Phenolic Extract*

The extractions of phenolic compounds in black soy milk were performed according to Ulyatu et al. [19] for phenolic content and antioxidant activity assay. A total of 2 milliliters of fermented black soy milk samples were extracted with 10 mL methanol (70%) using a water bath shaker (Sibata WS-240, Japan) at 120 rpm for 72 min at room temperature, followed by 24 h maceration at 4 °C in a dark room. Then, the extracts were centrifuged at 3000× *g* for 15 min, and the supernatant was filtered using Whatman paper no. 42. The natant was extracted again using the same method. Crude extract from the first and second extraction was mixed, and volume was measured and stored at −20 °C before being analyzed.

### *2.11. Folin-Ciocalteu Assay*

Folin–Ciocalteu assay was analyzed colorimetrically using the Folin–Ciocalteu method described by Ulyatu et al. [19]. Crude extracts were diluted four times before analysis. Two milliliters of samples were added with one milliliter of Folin-Ciocalteu reagent. The sample was vortexed and allowed to stand for one minute. Then, four milliliters of 15% sodium carbonate ($Na_2CO_3$) were added, and the samples were vortexed again. Samples were allowed to stand in a dark room for 2 h at room temperature. The absorbance was measured at 760 nm using a UV-Vis spectrophotometer (Thermo Fisher Scientific Genesys 150, Waltham, MA, USA). Gallic acid was used as the standard, and methanol was used as blank. The results are expressed in mg Gallic Acid Equivalent (GAE) per 100 mL fermented black soy milk.

### *2.12. Antioxidant Activity Assay*

The antioxidant activity of fermented black soy milk was determined colorimetrically by the ability of the extracts to scavenge the 2,2′-diphenyl-1-picrylhydrazyl (DPPH) radical described by Ulyatu et al. [19]. One milliliter of crude extracts was added with three

milliliters of 0.1 mM DPPH solution and was incubated for 30 min in a dark room at room temperature. The absorbance was measured using the UV-Vis spectrophotometer at 515 nm. Methanol was used as a control, and the radical scavenging activity was determined using the following equation.

$$Radical\ Scavenging\ Activity\ (RSA) = \left(\frac{control\ absorbance - sampel\ absorbance}{control\ absorbance}\right) \times 100\% \quad (1)$$

### 2.13. Isoflavone Aglycone Daidzein and Genistein Analysis

Determination of isoflavone aglycone daidzein and genistein was performed according to Sulistyowati et al. [20] using High-Performance Liquid Chromatography. Fermented black soy milk was freeze-dried before analysis. One gram of freeze-dried samples was extracted with 10 mL methanol (50%). Extraction was performed using an ultrasonic bath for 30 min. The samples were then centrifuged at 3000 rpm for 15 min, and the supernatant was filtered two times using Whatman paper no.1 and a syringe filter of 0.45 µm before being injected into HPLC systems. Samples were analyzed using HPLC (LC-20AD, Shimadzu, Japan) equipped with an autosampler (SIL-20A HT, Shimadzu, Japan), quaternary pump, PDA detector (CTO-20A, Shimadzu, Japan), degassing unit (DGU-20A SR, Shimadzu, Japan). The column used was Sun Fire TMC reverse phase C-18 column (150 mm × 4.6 mm, 5 µm). Meanwhile, the mobile phase consists of methanol (solvent A) and 0.1% acetic acid in water (solvent B). The flow rate was set isocratic at 1 mL·min$^{-1}$ with the ratio of solvent A:B = 53:47 for 15 min at 30 °C. The detector was set at 254 nm, respectively. The quantity of daidzein and genistein was calculated based on a standard curve.

### 2.14. Determination of β-Glucosidase Activity

The β-glucosidase activity was determined colorimetrically by the rate of hydrolysis of substrate p-nitrophenyl-α-D-glucopyranoside (pNPG), according to Djafaar et al. [21]. Fermented black soy milk was centrifuged at 4000 rpm, 4 °C for 15 min, and the supernatant was used as the crude enzyme. Then, 500 µL crude enzyme was added into 1 mL of 5 mM pNPG prepared in a 100 mM sodium phosphate buffer (pH 7) and incubated at 37 °C for 30 min. One milliliter of cold sodium carbonate was added to stop the reaction. The absorbance was measured using a spectrophotometer UV-Vis at 401 nm, and p-nitrophenol was used as standard. One unit of the enzyme was defined as the amount of enzyme releasing one µmol of p-nitrophenol from the substrate p-NPG per min under assay conditions.

### 2.15. Data Analysis

The experiment was performed in two trials, each with two replicates of analysis. Data are expressed as mean ± standard deviation (SD). Data were analyzed using one-way ANOVA followed by Duncan's Multiple Range Test with a significance level of $p < 0.05$ and performed using IBM SPSS Statistic 20 software (IBM, Armonk, NY, USA) [22]. The *p*-values below 0.05 were considered statistically significant.

## 3. Results

### 3.1. Chemical Properties of Black Soy Milk

The chemical properties such as proximate, minerals, sugar profile, gallic acid equivalent, isoflavone aglycone, and amino acid composition of black soy milk were studied before fermentation was conducted. Table 2 shows the chemical properties of black soy milk. Black soy milk contains a high amount of protein and sucrose. This protein and sugar might be useful for LAB growth as nitrogen and carbon sources, respectively. Some minerals such as iron, zinc, magnesium, and manganese, which are needed for LAB growth, are also available in black soy milk. Moreover, black soy milk already contains isoflavone aglycone daidzein and genistein. Table 3 presents the amino acid profile of black soy milk. Glutamic acid, L-arginine, and L-aspartic acid were the major amino acids in black soy milk. Essential amino acid L-arginine was found higher compared to other essential amino acids. Moreover, essential amino acids L-phenylalanine and L-leucine were also found in an abundant amount.

**Table 2.** Chemical properties of black soy milk.

| Parameters | Black Soy Milk |
|---|---|
| Moisture (%) | 92.45 ± 0.03 |
| Crude fat (g·100 mL$^{-1}$) | 0.18 ± 0.03 |
| Crude protein (g·100 mL$^{-1}$) | 3.78 ± 0.05 |
| Ash (g·100 mL$^{-1}$) | 0.41 ± 0.01 |
| Iron (µg·mL$^{-1}$) | 6.11 ± 0.03 |
| Zinc (µg·mL$^{-1}$) | 4.66 ± 0.02 |
| Magnesium (µg·mL$^{-1}$) | 246.98 ± 0.04 |
| Manganese (µg·mL$^{-1}$) | 2.98 ± 0.01 |
| Carbohydrate (g·100 mL$^{-1}$) | 95.61 ± 0.02 |
| Fructose (g·100 mL$^{-1}$) | 0.48 ±0.01 |
| Glucose (g·100 mL$^{-1}$) | n.d. [1] |
| Sucrose (g·100 mL$^{-1}$) | 3.93 ± 0.01 |
| Maltose (g·100 mL$^{-1}$) | n.d. [1] |
| Lactose (g·100 mL$^{-1}$) | n.d. [1] |
| Gallic acid equivalent (mg GAE·100 mL$^{-1}$) | 33.26 ± 3.55 |
| Daidzein (µg·mL$^{-1}$) | 10.39 ± 0.07 |
| Genistein (µg·mL$^{-1}$) | 6.14 ± 0.01 |

[1] n.d. = not detected. Limit of detection (LOD) = 0.06 g·100 mL$^{-1}$. Values are expressed as mean ± SD ($n$ = 4).

**Table 3.** Amino acid composition of black soy milk.

| Amino Acids | Concentration (g·100 mL$^{-1}$) |
|---|---|
| L-Serine | 0.38 ± 0.01 |
| Glutamic acid | 0.99 ± 0.01 |
| L-phenylalanine | 0.46 ± 0.01 |
| L-Isoleucine | 0.25 ± 0.01 |
| L-Valine | 0.25 ± 0.01 |
| L-Alanine | 0.21 ± 0.01 |
| L-Arginine | 0.60 ± 0.01 |
| Glycine | 0.28 ± 0.01 |
| L-Lysine | 0.27 ± 0.01 |
| L-Aspartic acid | 0.56 ± 0.01 |
| L-Leucine | 0.44 ± 0.01 |
| L-Tyrosine | 0.28 ± 0.01 |
| L-Proline | 0.28 ± 0.01 |
| L-Threonine | 0.28 ± 0.01 |
| L-Histidine | 0.22 ± 0.01 |
| L-Cysteine | 0.09 ± 0.01 |
| L-Methionine | 0.06 ± 0.01 |
| L-Tryptophan | 0.05 ± 0.01 |

Values are expressed as mean ± SD ($n$ = 2).

### *3.2. Fermentation of Black Soy Milk by Indonesian Indigenous LAB*

Black soy milk contains an abundant nutrient that has potential as a carbon and nitrogen source and growth factor for LAB growth. Figure 1 shows the growth and acid production of Indonesian LAB during black soy milk fermentation. All strains could grow well in black soy milk medium, reaching 9 log CFU·mL$^{-1}$. Both *L. plantarum* strains showed a similar growth pattern that reached the stationary phase after 18 h of fermentation. Meanwhile, *S. thermophilus* Dad 11 reached the stationary phase after 12 h of fermentation. The metabolic activities during LAB growth led to the production of acid. An increase in titratable acidity and decreased pH value indicated the acid production during fermentation of black soy milk. All strains exhibited an increase in titratable acidity throughout fermentation even though the pH value did not significantly increase after 18 h for *L. plantarum* WGK 4 and 12 h for *S. thermophilus* Dad 11 and *L. plantarum* Dad 13. *L. plantarum* WGK 4 produces the highest acid, 1.50%, compared to the other two strains.

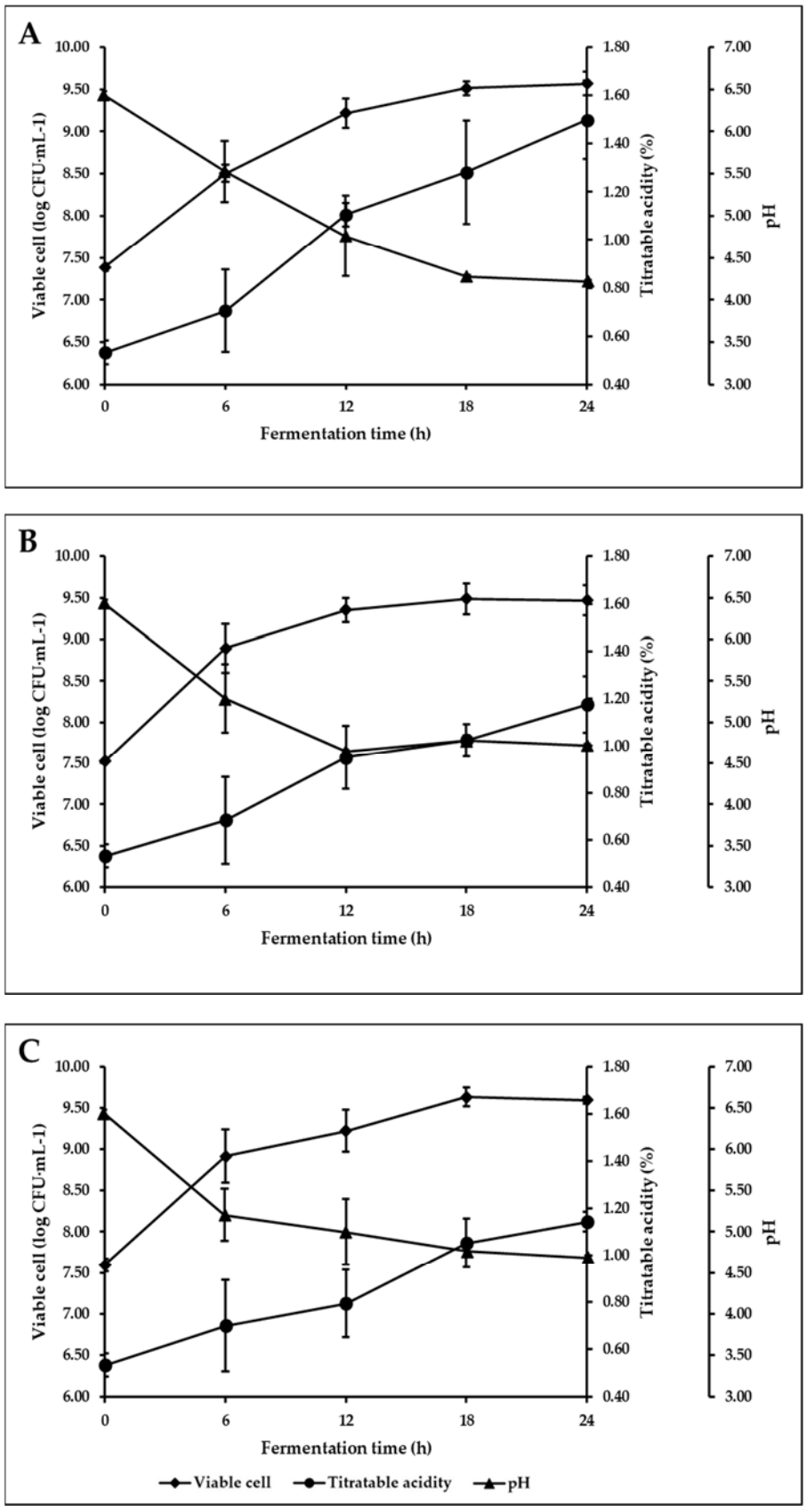


**Figure 1.** Growth and acid production ($n$ = 4) of: (**A**) *L. plantarum* WGK 4, (**B**) *S. thermophilus* Dad 11, and (**C**) *L. plantarum* Dad 13 during fermentation of black soy milk (24 h, 37 °C).

The organic acid composition (lactic and acetic acid) was further analyzed during 18 h of fermentation of black soy milk since the pH value at 24 h of fermentation was not significantly different compared to the previous hour. Table 4 describes the lactic and acetic acid content in black soy milk during fermentation by Indonesian indigenous LAB. Lactic acid was the dominant acid produced. Acetic acid was not detected until 18 h of fermentation.

**Table 4.** Lactic and acetic acid of black soy milk during fermentation by Indonesian indigenous LAB (24 h, 37 °C).

| Strains | Fermentation Time (h) | Lactic Acid (mg·100 mL$^{-1}$) | Acetic Acid (mg·100 mL$^{-1}$) |
|---|---|---|---|
| *L. plantarum* WGK 4 | 0 | n.d. [1] | n.d. [1] |
| | 6 | 0.20 ± 0.01 | n.d. [1] |
| | 12 | 0.72 ± 0.01 | n.d. [1] |
| | 18 | 1.14 ± 0.01 | 0.03 ± 0.01 |
| *S. thermophilus* Dad 11 | 0 | n.d. [1] | n.d. [1] |
| | 6 | 0.40 ± 0.01 | n.d. [1] |
| | 12 | 0.73 ± 0.01 | n.d. [1] |
| | 18 | 0.93 ± 0.01 | 0.07 ± 0.01 |
| *L. plantarum* Dad 13 | 0 | n.d. [1] | n.d. [1] |
| | 6 | 0.36 ± 0.01 | n.d. [1] |
| | 12 | 0.52 ± 0.01 | n.d. [1] |
| | 18 | 1.03 ± 0.01 | 0.05 ± 0.01 |

[1] n.d. = not detected. Limit of detection (LOD) = 0.41 µg·100 mL$^{-1}$ (lactic acid) and 0.04 µg·100 mL$^{-1}$ (acetic acid). Values are expressed as mean ± SD ($n$ = 2).

One of the requirements for LAB to grow is sufficient carbon sources and the ability to utilize those carbon sources. Table 5 displays the sugar composition throughout black soy milk fermentation. A decrease in sucrose showed that Indonesian indigenous LAB could utilize this sugar. Sucrose was broken down into fructose and glucose. There was also a decrease in fructose content during fermentation. This points out that Indigenous LAB utilizes this sugar as a carbon source for their growth and metabolic activities. Glucose was mainly not detected during fermentation. It might be because the LAB rapidly consumed it.

**Table 5.** Sugars profile of black soy milk during fermentation by Indonesian indigenous LAB (18 h, 37 °C).

| Strains | Fermentation Time (h) | Fructose (g·100 mL$^{-1}$) | Glucose (g·100 mL$^{-1}$) | Sucrose (g·100 mL$^{-1}$) | Maltose (g·100 mL$^{-1}$) | Lactose (g·100 mL$^{-1}$) |
|---|---|---|---|---|---|---|
| *L. plantarum* WGK 4 | 0 | 0.48 ± 0.01 | n.d. [1] | 3.94 0.01 | n.d. [1] | n.d. [1] |
| | 6 | 0.57 ± 0.01 | 0.70 ± 0.01 | 2.18 ± 0.01 | n.d. [1] | n.d. [1] |
| | 12 | 0.32 ± 0.01 | n.d. [1] | 0.54 ± 0.01 | n.d. [1] | n.d. [1] |
| | 18 | n.d. [1] | n.d. [1] | n.d. [1] | n.d. [1] | n.d. [1] |
| *S. thermophilus* Dad 11 | 0 | 0.48 ± 0.01 | n.d. [1] | 3.94 ± 0.01 | n.d. [1] | n.d. [1] |
| | 6 | 0.43 ± 0.01 | n.d. [1] | 1.71 ± 0.01 | n.d. [1] | n.d. [1] |
| | 12 | n.d. [1] | n.d. [1] | 0.46 ± 0.01 | n.d. [1] | n.d. [1] |
| | 18 | n.d. [1] | n.d. [1] | n.d. [1] | n.d. [1] | n.d. [1] |
| *L. plantarum* Dad 13 | 0 | 0.48 ± 0.01 | n.d. [1] | 3.94 ± 0.01 | n.d. [1] | n.d. [1] |
| | 6 | n.d. [1] | n.d. [1] | 1.69 ± 0.01 | n.d. [1] | n.d. [1] |
| | 12 | 0.28 ± 0.01 | n.d. [1] | 0.73 ± 0.01 | n.d. [1] | n.d. [1] |
| | 18 | n.d. [1] | n.d. [1] | n.d. [1] | n.d. [1] | n.d. [1] |

[1] n.d. = not detected. Limit of detection (LOD) = 0.06 g·100 mL$^{-1}$. Values are expressed as mean ± SD ($n$ = 2).

### *3.3. Antioxidant Properties of Black Soy Milk during Fermentation by Indonesian Indigenous LAB*

Plant-based milk, such as black soy milk, is a primary source of phenolic compounds. Phenolic compounds are known to have beneficial effects, such as antioxidants. Table 6 shows the Folin–Ciocalteu assay and antioxidant activity of fermented black soy milk. Black soy milk fermented with either *L. plantarum* WGK 4 or Dad 13 significantly increased the gallic acid equivalent until 18 h of fermentation. It decreased after 24 h of fermentation, while *S. thermophilus* Dad 11 exhibited

increased gallic acid equivalent content until 24 h of fermentation but not as much as two other strains. Nevertheless, for antioxidant activity, fermented black soy milk by *L. plantarum* WGK 4 and *S. thermophilus* Dad 11 showed a similar pattern in enhancing antioxidants, while *L. plantarum* Dad 13 exhibited increased DPPH-scavenging activity until 18 h but decreased afterward.

**Table 6.** Folin–Ciocalteu assay and DPPH-scavenging activity of black soy milk during fermentation by Indonesian indigenous LAB (24 h, 37 °C).

| Strains | Fermentation Time (h) | Folin–Ciocalteu Assay (mg GAE·mL$^{-1}$) | DPPH-Scavenging Activity (%) |
|---|---|---|---|
| *L. plantarum* WGK 4 | 0 | 33.26 ± 3.55 $^{a}$ | 24.90 ± 2.50 $^{a}$ |
| | 6 | 32.04 ± 0.84 $^{a}$ | 30.37 ± 1.30 $^{b}$ |
| | 12 | 40.17 ± 3.05 $^{b}$ | 29.51 ± 0.26 $^{b}$ |
| | 18 | 44.44 ± 2.56 $^{c}$ | 36.37 ± 0.59 $^{c}$ |
| | 24 | 38.27 ± 1.89 $^{b}$ | 38.20 ± 0.55 $^{c}$ |
| *S. thermophilus* Dad 11 | 0 | 33.26 ± 3.55 $^{ab}$ | 24.90 ± 2.50 $^{ab}$ |
| | 6 | 30.17 ± 0.37 $^{a}$ | 24.11 ± 0.72 $^{ab}$ |
| | 12 | 31.25 ± 0.89 $^{a}$ | 22.83 ± 2.35 $^{a}$ |
| | 18 | 33.70 ± 2.25 $^{ab}$ | 27.15 ± 1.56 $^{b}$ |
| | 24 | 36.11 ± 4.38 $^{b}$ | 32.04 ± 3.44 $^{c}$ |
| *L. plantarum* Dad 13 | 0 | 33.98 ± 3.97 $^{a}$ | 24.90 ± 2.50 $^{a}$ |
| | 6 | 32.82 ± 0.70 $^{a}$ | 25.74 ± 0.78 $^{a}$ |
| | 12 | 32.41 ± 0.82 $^{a}$ | 29.27 ± 2.44 $^{b}$ |
| | 18 | 37.66 ± 1.03 $^{b}$ | 36.87 ± 0.68 $^{c}$ |
| | 24 | 32.20 ± 2.61 $^{a}$ | 31.22 ± 2.93 $^{b}$ |

Values are expressed as mean ± SD ($n$ = 4). Values of each strain with different superscripts ($^{a,b,c}$) are significantly different ($p < 0.05$) by Duncan's multiple range test.

### *3.4. Isoflavone Aglycone Liberation throughout Black Soy Milk Fermentation by Indonesian Indigenous LAB*

The increased antioxidant activity of black soy milk during fermentation might be due to the liberation of isoflavone aglycone from its glucoside form in β-glucosidase. The current results showed an increased daidzein and genistein concentration in black soy milk during fermentation by all LAB strains, along with an increase in β-glucosidase activity (Table 7). Daidzein concentration was increased by about 61%, 107%, and 103% for black soy milk fermented with *L. plantarum* WGK 4, *S. thermophilus* Dad 11, and *L. plantarum* Dad 13, respectively. Meanwhile, genistein concentration was increased by about 81%, 132%, and 108% for black soy milk fermented with *L. plantarum* WGK 4, *S. thermophilus* Dad 11, and *L. plantarum* Dad 13, respectively. Nevertheless, isoflavone aglycone decreased after 24 h fermentation, even though the β-glucosidase activity still increased in black soy milk fermented with *S. thermophilus* Dad 11.

**Table 7.** Isoflavone aglycone (daidzein and genistein) and β-glucosidase activity of black soy milk during fermentation by Indonesian indigenous LAB (24 h, 37 °C).

| Strains | Fermentation Time (h) | Daidzein (μg·mL$^{-1}$) | Genistein (μg·mL$^{-1}$) | β-Glucosidase Activity (mU·mL$^{-1}$) |
|---|---|---|---|---|
| *L. plantarum* WGK 4 | 0 | 10.39 ± 0.07 $^{a}$ | 6.14 ± 0.01 $^{a}$ | - |
| | 6 | 13.97 ± 0.67 $^{b}$ | 7.08 ± 0.01 $^{b}$ | 25.28 ± 0.40 $^{a}$ |
| | 12 | 16.04 ± 0.01 $^{cd}$ | 9.72 ± 0.02 $^{c}$ | 27.43 ± 3.44 $^{a}$ |
| | 18 | 15.81 ± 0.05 $^{c}$ | 10.56 ± 0.01 $^{d}$ | 24.09 ± 1.86 $^{a}$ |
| | 24 | 16.69 ± 0.12 $^{d}$ | 11.03 ± 0.01 $^{e}$ | 32.57 ± 1.34 $^{b}$ |
| *S. thermophilus* Dad 11 | 0 | 10.39 ± 0.07 $^{a}$ | 6.14 ± 0.01 $^{a}$ | - |
| | 6 | 17.03 ± 0.09 $^{b}$ | 10.21 ± 0.07 $^{b}$ | 23.62 ± 2.90 $^{a}$ |
| | 12 | 20.34 ± 0.01 $^{d}$ | 13.14 ± 0.06 $^{d}$ | 36.69 ± 5.18 $^{b}$ |
| | 18 | 21.55 ± 0.11 $^{e}$ | 14.24 ± 0.09 $^{e}$ | 34.84 ± 1.41 $^{b}$ |
| | 24 | 19.48 ± 0.01 $^{c}$ | 11.40 ± 0.01 $^{c}$ | 48.32 ± 1.04 $^{c}$ |
| *L. plantarum* Dad 13 | 0 | 10.39 ± 0.07 $^{a}$ | 6.14 ± 0.01 $^{a}$ | - |
| | 6 | 16.94 ± 0.65 $^{b}$ | 10.62 ± 0.02 $^{b}$ | 25.18 ± 5.81 $^{a}$ |
| | 12 | 18.77 ± 0.09 $^{c}$ | 11.30 ± 0.02 $^{c}$ | 36.55 ± 3.03 $^{b}$ |
| | 18 | 20.09 ± 0.04 $^{d}$ | 12.75 ± 0.01 $^{e}$ | 45.05 ± 4.53 $^{c}$ |
| | 24 | 18.89 ± 0.02 $^{c}$ | 12.20 ± 0.10 $^{d}$ | 35.37 ± 1.00 $^{b}$ |

Values are expressed as mean ± SD ($n$ = 4). Values of each strain with different superscripts ($^{a,b,c,d,e}$) are significantly different ($p < 0.05$) by Duncan's multiple range test.

## 4. Discussion

The main aim of the current study was to investigate the ability of Indonesian LAB to enhance the antioxidant properties of black soy milk during fermentation. The enhancement of the antioxidant activities through fermentation can be developed into a functional food product to diversify black soybean utilization. The current LAB needs to grow well in a black soy milk medium to enhance its antioxidant properties. Lactic acid bacteria growth depends on the nutrients available in the medium and the LAB's ability to utilize those nutrients [23].

Carbon source is one of the primary nutrients needed for LAB growth. Generally, the major sugar in soybean is sucrose [24]. This finding is in agreement with the current study. Moreover, sucrose concentration in black soy milk is higher than glucose concentration in the MRS medium, which is 20 g·L$^{-1}$. LAB could use sucrose as a carbon source, supported by the decreased sucrose concentration during fermentation of black soy milk (Table 6). Additionally, Baú et al. [25] also found that stachyose was the second major sugar found in soy milk. Meanwhile, the major sugar in hazelnut milk and *kerandang* milk was glucose [26] and raffinose [27], respectively. It is possible that LAB could also utilize these sugars after utilizing simple sugars such as sucrose. Singh et al. [28] stated that besides sucrose, *L. plantarum* C6 could utilize other sugars, such as stachyose and raffinose, as a substrate for its growth by producing α-galactosidase. Moreover, the reduction of sucrose was faster than stachyose and raffinose. In addition, Baú et al. [25] found that sucrose was reduced at an early stage of fermentation, while stachyose and raffinose were decreased after the sucrose was not detected. In addition to carbon sources, nitrogen sources are also an essential nutrient for LAB growth.

This study also found that black soy milk contains a high protein compared to other milk. Chalupa-Krebzdak et al. [29] reported that the protein content in whole bovine milk, soy milk, coconut milk, and cashew milk is 3.15%, 2.28%, 1.28%, and 1.31%, respectively. Additionally, Kundu et al. [30] found that the protein content of almond milk was 1.308%. Lactic acid bacteria might utilize small peptides and free amino acids as a nitrogen source. Singh et al. [28] mentioned that several lactobacilli have proteolytic activity by producing extracellular proteinase to hydrolyze soy protein in soy milk. Moreover, Boulay et al. [31] stated that *S. thermophilus* LMD-9 could hydrolyze soy milk protein, and the cell wall protease PrtS plays an important role. Additionally, LAB also needs some specific amino acids for their growth. Arginine, leucine, valine, glutamic acid, and cysteine are needed for *L. plantarum* growth; meanwhile, cysteine or methionine, and histidine are needed for *S. thermophilus* growth [32]. This study showed that black soy milk contained those amino acids, especially glutamic acid and arginine. For its metabolic activity, some LAB needs minerals for the enzymatic reaction. The minerals usually required are Fe, Mg, Mn, and Zn, but this requirement is basically strain-dependent [33]. This study found that black soy milk has these minerals. Furthermore, the Mg and Mn concentrations in black soy milk were on par with the MRS medium. The findings indicate that the nutrient in black soy milk might be enough for Indonesian indigenous LAB growth.

Indonesian indigenous LAB demonstrated good growth in black soy milk medium. Results showed that *S. thermophilus* Dad 11 was on par with the viable cell of *S. thermopilus* S10, which increased 2 log cycles, reaching 9–9.5 log CFU·mL$^{-1}$, after 24 h fermentation of black soy milk, reported by Lee et al. [12]. Even the current study strain, *S. thermophilus* Dad 11, has a higher viable cell in fermented black soy milk (Figure 1) compared to the viable cell of *S. thermophilus* ATCC BAA-250 after 24 h of soy milk fermentation, which was 8.58 log CFU·mL$^{-1}$, demonstrated by Liu et al. [34]. For *L. plantarum*, the results (Figure 1) were higher than the viable cell of *L. plantarum* 70810, 8.57 log CFU·mL$^{-1}$, after fermenting soy milk for 12 h [35] and on par but reached faster with the cell counts of *L. plantarum* LMG6940 during fermentation of soy milk, which was increased from 7.63 log CFU·mL$^{-1}$ to 9.52 log CFU·mL$^{-1}$ after 48 h [36]. Additionally, *L. plantarum* WGK 4 showed higher black soy milk growth than jack bean milk, which only increased 1 log cycle after 24 h of fermentation at 37 °C [15]. Furthermore, *L. plantarum* Dad 13 and *S. thermophilus* Dad 11 showed better growth in black soy milk than in skimmed milk, which only increased 1 log cycle after 24 h of fermentation at 37 °C [14].

Not only do they grow well, but Indonesian indigenous LAB also showed good metabolic activity, proven by the acid production. These LAB strains have a higher acidification rate than previous studies, especially *L. plantarum* WGK 4 (Figure 1). Kuda et al. [37] developed several *L. plantarum* strains from plants of the coastal Satoumi regions as starter culture in soy milk fermentation. The pH and lactic acid concentrations range around 5.1–5.4 and 3.0–5.7 mg·mL$^{-1}$, respectively. Kim et al. [38] introduced soy yogurt fermented by *Leuconostoc mesenteroides* and *L. plantarum* from kimchi, wherein the pH and titratable acidity after 24 h were around 4.5% and 0.8%. Moreover, Liu et al. [34] demonstrated the acidification rate of *S. thermophilus* ATCC BAA-250 during soy milk fermentation. The pH did not decrease much at 12 to 24 h, around 5.22–5.18. Wardani et al. [16] found

that milk's titratable acidity and pH after fermentation using *L. plantarum* Dad 13 at 37 °C were 0.46% and 5.27, respectively. This research found that *L. plantarum* WGK 4 has higher acid production than *S. thermophilus* Dad 11 and *L. plantarum* Dad 13 (Figure 1). It might be because *S. thermophilus* Dad 11 and *L. plantarum* Dad 13 were isolated from Dadih (Indonesian fermented buffalo milk). Therefore, the ability to utilize the nutrient in plant-based milk was lower than *L. plantarum* WGK 4, isolated from red lima bean soaking water in tempeh production. Indonesian indigenous LAB showed better growth and acid production due to the nutrients available in black soy milk. In addition to utilizing the available sugars in the black soybean milk, there is also a possibility that LAB used glucose moiety in isoflavone glucoside and, therefore, might enhance the antioxidant properties.

The gallic acid equivalent and antioxidant activity increased during fermentation of black soy milk by Indonesian LAB. Consistent with current results, Ulyatu et al. [19] also noted an increase in gallic acid equivalent content and DPPH-scavenging activity during fermentation of sesame milk using *L. plantarum* Dad 13. A similar increment of both gallic acid equivalent content and antioxidant activity was described by Lee et al. [12] in fermented black soy milk by *S. thermophilus* S10. Gallic acid equivalent content and DPPH scavenging activity of black soy milk increased significantly throughout 24 h fermentation using single culture of *S. thermophilus* S10. Moreover, the current study showed higher DPPH-scavenging activity than the finding of Zhao et al. [11], which reported that soy milk fermented with *L. acidophilus* CSCC 2400, *L. paracasei* CSCC 279, *L. zeae* ASCC 15820, and *L. rhamnosus* WQ2 until the pH reached 4.55 have DPPH-scavenging activity around 15–20%. Salar et al. [39] stated that lactic acid bacteria could produce β-glucosidase during fermentation. This enzyme can hydrolyze glucoside phenolic into free phenols, thus, increasing the gallic acid equivalent content of black soy milk. Moreover, Fitrotin [13] mentioned that during sesame milk fermentation, lactic acid bacteria could produce β-glucosidase to hydrolyze isoflavone glucoside into its aglycone form. Aglycone phenolic compound has more reactivity to reduce both phosphomolybdate phosphotungstate complex in Folin–Ciocalteu reagent and DPPH radical due to the more available hydroxyl groups; therefore, increasing the gallic acid equivalent content and antioxidant activity.

Nevertheless, this study also discovered a decrease in gallic acid equivalent and antioxidant activity at 24 h of fermentation in some strains. The gallic acid equivalent content of black soy milk after 24 h of fermentation by *L. plantarum* WGK 4 and *L. plantarum* Dad 13 exhibited a lower concentration than 18 h of fermentation. Phenolic compounds have been reported to have an inhibitory effect on LAB, and *L. plantarum* possessed the ability to degrade those phenolic compounds as a stress response [40]. It is possible that at 24 h of fermentation, *L. plantarum* strains shifted to degrade phenolic compounds due to stress response. Along with the decreased gallic acid equivalent, this study found a decreased DPPH-scavenging activity for black soy milk fermented by *L. plantarum* Dad 13 but not for *L. plantarum* WGK 4. It points out the possibility of other antioxidant compounds released during black soy milk fermentation, such as bioactive peptides. Yusuf et al. [41] discovered an increased antioxidant activity of 10 *Lactobacillus* spp. isolated from Indonesian kefir grains due to the liberation of the bioactive peptide by proteolytic activities during milk fermentation.

Fermentation by Indonesian indigenous LAB enhances the antioxidant properties of black soy milk. This study demonstrated increased isoflavone aglycone daidzein and genistein concentration and β-glucosidase activity. Lee et al. [42] also discovered an increased daidzein and genistein concentration of several cultivars of fermented soybean powder hydrolysate milk by *L. plantarum* P1201 for 48 h at 35 °C. Current findings were higher than the results reported by Hati et al. [7], which found the daidzein and genistein concentration in soy milk fermented with several *L. rhamnosus* and *L. casei* strain for 12 h at 37 °C was around 0.81–0.98 mg·100 $mL^{-1}$ and 1.13–1.93 mg·100 $mL^{-1}$, respectively. They also stated that the β-glucosidase activity was varied among strains which corresponds to these findings. The results were also higher than the daidzein and genistein concentration in soy milk fermented with *L. plantarum* for 24 h, 0.423 mg·100 $mL^{-1}$ and 0.753 mg·100 $mL^{-1}$, respectively [10]. Moreover, Lee et al. [12] also reported increased β-glucosidase activity, around 60 UA·$g^{-1}$, along with increased daidzein and genistein content, reaching 119.39 mg·100 $g^{-1}$ and 191.38 mg·100 $g^{-1}$, respectively, during fermentation of black soy milk by *S. thermophilus* S10 at 37 °C. The β-glucosidase activity in fermented black soy milk by indigenous LAB was higher than the β-glucosidase activity in *kerandang* extract for *L. plantarum* T33, which was 20 mU·mL $culture^{-1}$, but lower than the β-glucosidase activity from *L. plantarum-pentosus*, T14 558 mU·mL $culture^{-1}$ [21]. Nevertheless, the current study also found a decrease in isoflavone aglycone concentration at 24 h fermentation even though the β-glucosidase activity was still increasing in black soy milk fermented by *S. thermophilus* Dad 11. Cairns et al. [43] mentioned that β-glucosidase also catalyzes glycosylation reaction or reverse hydrolysis. Thus, reverse conversion might happen. The current study demonstrated that

Indonesian indigenous LAB could produce β-glucosidase to break down isoflavone glucoside into isoflavone aglycone and glucose. Glucose was utilized as a carbon source for LAB's growth and metabolic activity. Meanwhile, the isoflavone aglycone enhances antioxidant properties.

## 5. Conclusions

Indonesian indigenous LAB, *L. plantarum* WGK 4, *S. thermophilus* Dad 11, and *L. plantarum* Dad 13 grow well in black soy milk medium. These LAB could utilize the nutrient contained in black soy milk for its growth and metabolic activity. Additionally, they could produce β-glucosidase to hydrolyze isoflavone glucoside into glucose and isoflavone aglycone daidzein and genistein. Thus, it enhanced the antioxidant properties, such as gallic acid equivalent content and DPPH-scavenging activity, of black soy milk. It points out that Indonesian indigenous LAB has a promising potential to be used as a starter culture to develop fermented non-dairy milk, such as black soy milk, as functional food products. Further studies are necessary to investigate other antioxidant properties and other antioxidant compounds that might be released during fermentation. Product formulation and sensory evaluation, including flavor, taste, and physical properties, are also needed to develop fermented black soy milk into a functional food product.

**Author Contributions:** Conceptualization, M.N.C., E.S.R., R.Y. and T.U.; methodology, B.Y.L., M.N.C. and T.U.; software, B.Y.L.; validation, M.N.C., E.S.R., R.Y. and T.U.; formal analysis, B.Y.L.; investigation, B.Y.L., M.N.C. and T.U.; resources, M.N.C., E.S.R., R.Y. and T.U.; data curation, B.Y.L.; writing—original draft preparation, B.Y.L. and T.U.; writing—review and editing, M.N.C., E.S.R. and R.Y.; visualization, B.Y.L. and T.U.; supervision, M.N.C., E.S.R., R.Y., T.U.; project administration, T.U.; funding acquisition, T.U. All authors have read and agreed to the published version of the manuscript.

**Funding:** This research was funded by the Indonesia Ministry of Research and Higher Education (RISTEKDIKTI) through a competition-based research program 2020, grant number 2937/UN1.DITLIT/DITLIT/PT/2020. The APC was funded by Universitas Gadjah Mada.

**Institutional Review Board Statement:** Not applicable.

**Informed Consent Statement:** Not applicable.

**Data Availability Statement:** The data presented in this study are available on request from the corresponding author.

**Acknowledgments:** The authors acknowledge Saraswanti Indo Genetech, Bogor, Indonesia, Pri Haryono from the Center for Food and Nutrition Studies, Universitas Gadjah Mada, Indonesia, and Ashari from Laboratory of Chemical and Biochemical of Food and Agricultural Products, Department of Food and Agricultural Product Technology, Faculty of Agricultural Technology, Gadjah Mada University, Indonesia for providing technical assistant.

**Conflicts of Interest:** The authors declare no conflict of interest.

## References

1. Kim, M.Y.; Jang, G.Y.; Oh, N.S.; Baek, S.Y.; Lee, S.H.; Kim, K.M.; Kim, T.M.; Lee, J.; Jeong, H.S. Characteristics and in Vitro Anti-Inflammatory Activities of Protein Extracts from Pre-Germinated Black Soybean [*Glycine Max* (L.)] Treated with High Hydrostatic Pressure. *Innov. Food Sci. Emerg. Technol.* **2017**, *43*, 84–91. [CrossRef]
2. Lee, J.H.; Cho, K.M. Changes Occurring in Compositional Components of Black Soybeans Maintained at Room Temperature for Different Storage Periods. *Food Chem.* **2012**, *131*, 161–169. [CrossRef]
3. Kim, G.P.; Lee, J.; Ahn, K.G.; Hwang, Y.S.; Choi, Y.; Chun, J.; Chang, W.S.; Choung, M.G. Differential Responses of B Vitamins in Black Soybean Seeds. *Food Chem.* **2014**, *153*, 101–108. [CrossRef]
4. Joshi, J.; Rahal, A. Effect of Processing Method on Nutritional Quality of Black Soybean. *Asian J. Dairy Food Res.* **2018**, *37*, 162–164. [CrossRef]
5. Widowati, W.; Prahastuti, S.; Hidayat, M.; Hasianna, S.T.; Wahyudianingsih, R.; Eltania, T.F.; Azizah, A.M.; Aviani, J.K.; Subangkit, M.; Handayani, R.A.S.; et al. Detam 1 Black Soybean against Cisplatin-Induced Acute Ren Failure on Rat Model via Antioxidant, Antiinflammatory and Antiapoptosis Potential. *J. Tradit. Complement. Med.* **2022**, *12*, 426–435. [CrossRef]
6. Huang, G.; Cai, W.; Xu, B. Improvement in Beta-Carotene, Vitamin B2, GABA, Free Amino Acids and Isoflavones in Yellow and Black Soybeans upon Germination. *LWT Food Sci. Technol.* **2017**, *75*, 488–496. [CrossRef]
7. Hati, S.; Vij, S.; Singh, B.P.; Mandal, S. β-Glucosidase Activity and Bioconversion of Isoflavones during Fermentation of Soy milk. *J. Sci. Food Agric.* **2015**, *95*, 216–220. [CrossRef] [PubMed]

8. Hsiao, Y.H.; Ho, C.T.; Pan, M.H. Bioavailability and Health Benefits of Major Isoflavone Aglycones and Their Metabolites. *J. Funct. Foods* **2020**, *74*, 104164. [CrossRef]
9. Zuo, A.; Yanying, Y.; Li, J.; Binbin, X.; Xiongying, Y.; Yan, Q.; Shuwen, C. Study on the Relation of Structure and Antioxidant Activity of Isorhamnetin, Quercetin, Phloretin, Silybin and Phloretin Isonicotinyl Hydrazone. *Free Radic. Antioxid.* **2011**, *1*, 39–47. [CrossRef]
10. Ding, W.K.; Shah, N.P. Enhancing the Biotransformation of Isoflavones in Soy milk Supplemented with Lactose Using Probiotic Bacteria during Extended Fermentation. *J. Food Sci.* **2010**, *75*, 140–149. [CrossRef]
11. Zhao, D.; Shah, N.P. Changes in Antioxidant Capacity, Isoflavone Profile, Phenolic and Vitamin Contents in Soy milk during Extended Fermentation. *LWT Food Sci. Technol.* **2014**, *58*, 454–462. [CrossRef]
12. Lee, M.; Hong, G.E.; Zhang, H.; Yang, C.Y.; Han, K.H.; Mandal, P.K.; Lee, C.H. Production of the Isoflavone Aglycone and Antioxidant Activities in Black soy milk Using Fermentation with *Streptococcus thermophilus* S10. *Food Sci. Biotechnol.* **2015**, *24*, 537–544. [CrossRef]
13. Fitrotin, U.; Utami, T.; Hastuti, P.; Santoso, U. Antioxidant Properties of Fermented Sesame Milk Using *Lactobacillus plantarum* Dad 13. *Int. Res. J. Biol.* **2015**, *4*, 56–61.
14. Utami, T.; Cindarbhumi, A.; Khuangga, M.C.; Rahayu, E.S.; Cahyanto, M.N.; Nurfiyani, S.; Zulaichah, E. Preparation of Indigenous Lactic Acid Bacteria Starter Cultures for Large Scale Production of Fermented Milk. In Proceedings of the 10th Asian Conference of Lactic Acid Bacteria, Yogyakarta, Indonesia, 10 February 2020.
15. Yudianti, N.F.; Yanti, R.; Cahyanto, M.N.; Rahayu, E.S.; Utami, T. Isolation and Characterization of Lactic Acid Bacteria from Legume Soaking Water of Tempeh Productions. In Proceedings of the 10th Asian Conference of Lactic Acid Bacteria, Yogyakarta, Indonesia, 10 February 2020.
16. Wardani, S.K.; Cahyanto, M.N.; Rahayu, E.S.; Utami, T. The Effect of Inoculum Size and Incubation Temperature on Cell Growth, Acid Production and Curd Formation during Milk Fermentation by *Lactobacillus plantarum Dad 13*. *Int. Food Res. J.* **2017**, *24*, 921–926.
17. AOAC International. *Official Methods of Analysis*, 21st ed.; AOAC International: Rockville, MD, USA, 2019.
18. Wasik, A.; McCourt, J.; Buchgraber, M. Simultaneous Determination of Nine Intense Sweeteners in Foodstuffs by High Performance Liquid Chromatography and Evaporative Light Scattering Detection-Development and Single-Laboratory Validation. *J. Chromatogr. A* **2007**, *1157*, 187–196. [CrossRef]
19. Ulyatu, F.; Pudji, H.; Tyas, U.; Umar, S. The Changes of Sesaminol Triglucoside and Antioxidant Properties during Fermentation of Sesame Milk by *Lactobacillus plantarum* Dad 13. *Int. Food Res. J.* **2015**, *22*, 1945–1952.
20. Sulistyowati, E.; Martono, S.; Riyanto, S.; Lukitaningsih, E. Development and Validation for Free Aglycones Daidzein and Genistein in Soybeans (*Glycine Max* (l.) Merr.) Using RP HPLC Method. *Int. J. Appl. Pharm.* **2019**, *11*, 138–142. [CrossRef]
21. Djafaar, T.F.; Santoso, U.; Cahyanto, M.N.; Takuya, S.; Rahayu, E.S.; Kosuke, N. Effect of Indigenous Lactic Acid Bacteria Fermentation on Enrichment of Isoflavone and Antioxidant Properties of Kerandang *(Canavalia Virosa)* Extract. *Int. Food Res. J.* **2013**, *20*, 2945–2950.
22. IBM SPSS Statistic 20. Available online: https://www.ibm.com/support/pages/downloading-ibm-spss-statistics-20 (accessed on 10 March 2021).
23. Berisvil, A.P.; Astesana, D.M.; Zimmermann, J.A.; Frizzo, L.S.; Rossler, E.; Romero-Scharpen, A.; Olivero, C.R.; Zbrun, M.V.; Signorini, M.L.; Sequeira, G.J.; et al. Low-Cost Culture Medium for Biomass Production of Lactic Acid Bacteria with Probiotic Potential Destined to Broilers. *FAVE Secc. Cienc. Vet.* **2020**, *20*, 1–9. [CrossRef]
24. Zheng, Y.; Li, L.; Jin, Z.; An, P.; Yang, S.T.; Fei, Y.; Liu, G. Characterization of Fermented Soy milk by Schleiferilactobacillus Harbinensis M1, Based on the Whole-Genome Sequence and Corresponding Phenotypes. *LWT Food Sci. Technol.* **2021**, *144*, 111237. [CrossRef]
25. Baú, T.R.; Garcia, S.; Ida, E.I. Changes in Soy milk during Fermentation with Kefir Culture: Oligosaccharides Hydrolysis and Isoflavone Aglycone Production. *Int. J. Food Sci. Nutr.* **2015**, *66*, 845–850. [CrossRef] [PubMed]
26. Bernat, N.; Cháfer, M.; Chiralt, A.; González-Martínez, C. Hazelnut Milk Fermentation Using Probiotic Lactobacillus Rhamnosus GG and Inulin. *Int. J. Food Sci. Technol.* **2014**, *49*, 2553–2562. [CrossRef]
27. Djaafar, T.F.; Cahyanto, M.N.; Santoso, U.; Rahayu, E.S. Growth of Indigenous Lactic Acid Bacteria *Lactobacillus plantarum-pentosus* T14 and *Lactobacillus plantarum-pentosus* T35 in Kerandang (*Canavalia Virosa*) Milk and Changes of Raffinose. *Malays. J. Microbiol.* **2013**, *9*, 213–218.
28. Singh, B.P.; Vij, S. α-Galactosidase Activity and Oligosaccharides Reduction Pattern of Indigenous Lactobacilli during Fermentation of Soy Milk. *Food Biosci.* **2018**, *22*, 32–37. [CrossRef]
29. Chalupa-Krebzdak, S.; Long, C.J.; Bohrer, B.M. Nutrient Density and Nutritional Value of Milk and Plant-Based Milk Alternatives. *Int. Dairy J.* **2018**, *87*, 84–92. [CrossRef]
30. Kundu, P.; Dhankhar, J.; Sharma, A. Development of Non Dairy Milk Alternative Using Soy milk and Almond Milk. *Curr. Res. Nutr. Food Sci.* **2018**, *6*, 203–210. [CrossRef]
31. Boulay, M.; al Haddad, M.; Rul, F. *Streptococcus thermophilus* Growth in Soya Milk: Sucrose Consumption, Nitrogen Metabolism, Soya Protein Hydrolysis and Role of the Cell-Wall Protease PrtS. *Int. J. Food Microbiol.* **2020**, *335*, 108903. [CrossRef]
32. Teusink, B.; Molenaar, D. Systems Biology of Lactic Acid Bacteria: For Food and Thought. *Curr. Opin. Syst. Biol.* **2017**, *6*, 7–13. [CrossRef]

33. Endo, A.; Dicks, L.M.T. Physiology of the LAB. In *Lactic Acid Bacteria: Biodiversity and Taxonomy*; Holzapfel, W.H., Wood, B.J.B., Eds.; John Wiley & Sons, Ltd.: Chichester, UK, 2014; pp. 13–20.
34. Liu, W.S.; Yang, C.Y.; Fang, T.J. Strategic Ultrasound-Induced Stress Response of Lactic Acid Bacteria on Enhancement of β-Glucosidase Activity for Bioconversion of Isoflavones in Soy milk. *J. Microbiol. Methods* **2018**, *148*, 145–150. [CrossRef]
35. Li, C.; Li, W.; Chen, X.; Feng, M.; Rui, X.; Jiang, M.; Dong, M. Microbiological, Physicochemical and Rheological Properties of Fermented Soy milk Produced with Exopolysaccharide (EPS) Producing Lactic Acid Bacteria Strains. *LWT Food Sci. Technol.* **2014**, *57*, 477–485.
36. Hong LE, P.; Parmentier, N.; Trung LE, T.; Raes, K. Evaluation of Using a Combination of Enzymatic Hydrolysis and Lactic Acid Fermentation for γ-Aminobutyric Acid Production from Soy milk. *LWT Food Sci. Technol.* **2021**, *142*, 111044. [CrossRef]
37. Kuda, T.; Kataoka, M.; Nemoto, M.; Kawahara, M.; Takahashi, H.; Kimura, B. Isolation of Lactic Acid Bacteria from Plants of the Coastal Satoumi Regions for Use as Starter Cultures in Fermented Milk and Soy milk Production. *LWT Food Sci. Technol..* **2016**, *68*, 202–207. [CrossRef]
38. Kim, H.J.; Han, M.J. The Fermentation Characteristics of Soy Yogurt with Different Content of D-Allulose and Sucrose Fermented by Lactic Acid Bacteria from Kimchi. *Food Sci. Biotechnol.* **2019**, *28*, 1155–1161. [CrossRef]
39. Salar, R.K.; Certik, M.; Brezova, V. Modulation of Phenolic Content and Antioxidant Activity of Maize by Solid State Fermentation with Thamnidium Elegans CCF 1456. *Biotechnol. Bioprocess Eng.* **2012**, *17*, 109–116. [CrossRef]
40. Rodríguez, H.; Curiel, J.A.; Landete, J.M.; de las Rivas, B.; de Felipe, F.L.; Gómez-Cordovés, C.; Mancheño, J.M.; Muñoz, R. Food Phenolics and Lactic Acid Bacteria. *Int. J. Food Microbiol.* **2009**, *132*, 79–90. [CrossRef] [PubMed]
41. Yusuf, D.; Nuraida, L.; Dewanti-Hariyadi, R.; Hunaef, D. In Vitro Antioxidant and α-Glucosidase Inhibitory Activities of Lactobacillus Spp. Isolated from Indonesian Kefir Grains. *Appl. Food Biotechnol.* **2021**, *8*, 39–46.
42. Lee, J.H.; Kim, B.; Hwang, C.E.; Haque, M.A.; Kim, S.C.; Lee, C.S.; Kang, S.S.; Cho, K.M.; Lee, D.H. Changes in Conjugated Linoleic Acid and Isoflavone Contents from Fermented Soy milks Using Lactobacillus Plantarum P1201 and Screening for Their Digestive Enzyme Inhibition and Antioxidant Properties. *J. Funct. Foods* **2018**, *43*, 17–28. [CrossRef]
43. Cairns, J.R.K.; Esen, A. β-Glucosidases. *Cell. Mol. Life Sci.* **2010**, *67*, 3389–3405. [CrossRef]