Journal of Tropical Biodiversity and Biotechnology
Volume 09, Issue 04 (2024): jtbb89003
DOI: 10.22146/jtbb.89003

Research Article

Characterization of Lactic Acid Bacteria Isolated from Soymilk and Its Growth in Soymilk By-product Medium for the Application in Soymilk Fermentation

Faizah Diah Retnowati[1], Yekti Asih Purwestri[1]*, Yuni Sine[1]
1)Faculty of Biology, Universitas Gadjah Mada, Jalan Teknika Selatan, Sendowo, Sinduadi, Kec. Mlati, Kabupaten Sleman, Daerah Istimewa Yogyakarta 55281
* Corresponding author, email: yekti@ugm.ac.id

Keywords:
Alternative growth medium
Lactic acid bacteria
Probiotic ability
Soymilk by-product
Submitted:
13 September 2023
Accepted:
03 May 2024
Published:
11 October 2024
Editor:
Furzani Binti Pa'ee

ABSTRACT
The separation phase of soy-product manufacturing, especially of soymilk production, involves using the liquid portion of soy while disposing primarily of the solid portion, known as soymilk by-product (SMB). The improper disposal of SMB can contribute to environmental concerns. As SMB contains many beneficial nutrients, this could serve as a valuable culture medium for lactic acid bacteria (LAB), especially considering the expense of the standard de Man, Rogosa, and Sharpe (MRS) medium. This study aimed to isolate and identify LAB from soymilk through 16S rRNA sequencing, assess the potential of SMB as a culture medium for LAB, and ferment soymilk with LAB cultured from SMB to demonstrate the probiotic capacity. The research identified a potential LAB as *Weissella confusa*. Effective cultivation of LAB was demonstrated at 2% SMB concentration, although LAB cultured with MRS medium yielded a higher colony count. Furthermore, fermentation of soymilk by the LAB isolates from SMB exhibited a positive probiotic ability, reaching 5.5×10^9 CFU/mL, with a lactic acid content of 0.27%.

INTRODUCTION
Soy is a staple food in many East Asian countries (Vanga & Raghavan 2018) and is widely consumed because of its versatility and nutritional value (Vanga & Raghavan 2018). Popular soy products include soymilk, tempeh, soy sauce, and various others that are enjoyed for their rich nutrient profiles. These products are favored for their abundance of proteins, dietary fiber-containing carbohydrates, vitamins, and minerals, all at a relatively low cost (Vanga & Raghavan 2018). With a protein content of up to 36.49% in its raw form, soy is one of the richest nonanimal sources of protein (Rizzo & Baroni 2018), making this a crucial dietary option for individuals adhering to vegan or vegetarian lifestyles.

Soymilk is one of the most successful soy-derived products and has been regularly consumed in most parts of the world as a replacement for dairy milk, often because of dietary preferences or intolerances. Lactose intolerance affects approximately 65% of the global population, according to Malik and Panuganti (2022), and is a prevalent issue with 75% to 100% of East Asian individuals experiencing lactose intolerance (Aydar et al. 2020).

However, the production of soy products, including soymilk, generates significant waste. During the processing of soybeans for products such as soymilk, the beans undergo soaking, grinding, separation, and pasteurization (Davy & Vuong 2022). During separation, the liquid soy portion is retained, whereas the solid by-product, known as soymilk by-product (SMB) or okara, is largely discarded (Davy & Vuong 2022).

Despite being discarded, SMB retains substantial nutrients, including proteins and carbohydrates, making this a potential resource for culturing lactic acid bacteria (LAB.) This is particularly valuable when considering the expense of standard culture media such as de Man, Ragosa, and Sharpe (MRS) medium (Hayek et al. 2019; Davy & Vuong 2022). Moreover, improper disposal of SMB poses environmental concerns because of decomposition processes, potentially impacting waterways during rainy weather.

Various studies, including those by Davy and Vuong (2022), Goda et al. (2011), and Kim (2019), have highlighted the potential of SMB as a culture medium for microorganisms such as *Pleurotus ostreatus* and *Phaeodactylum tricornutum*, yielding positive results. Aritonang et al. (2017) demonstrated the successful isolation of LAB from SMB, suggesting its suitability as a culture medium. The current study aims to explore the potential of using SMB for LAB culture media with a subsequent application in fermenting soymilk as a probiotic product to validate the probiotic efficacy of LAB cultured from SMB. Additionally, the study isolated LAB from fermented soymilk and applied 16S rRNA sequencing for their identification.

MATERIALS AND METHODS

Materials

For the preparation of soymilk and soymilk by-product (SMB), soybeans were sourced from Kranggan Traditional Market in the Special Region of Yogyakarta. For the isolation of LAB from soymilk, MRS broth medium and bacteriological grade agar powder was obtained from (HiMedia, Mumbai, India) and prepared using distilled water and saline as required.

For the morphological characterization of the isolated LAB, distilled water, crystal violet, iodine, 70% ethanol, safranin, microscope slides, and immersion oil were used for Gram staining. For the spore formation test, distilled water, filter paper, malachite green, safranin, microscope slides, and immersion oil were required. The motility test involved MRS broth, agar powder, and distilled water.

During the physiological and biochemical characterization, the catalase test required distilled water, microscope slides, and 3% hydrogen peroxide. Temperature and salt tolerance tests required MRS broth, NaCl, and distilled water.

The Bacteria DNA Isolation Kit by Geneaid was used for genomic DNA extraction. Electrophoresis required agarose, 1 × Tris borate-EDTA buffer (TBE), FloroSafe DNA Stain (1st BASE), 1 Kb Plus DNA ladder, and loading dye. PCR used universal forward and reverse primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1427R (5′-GGTTACCTTGTTACGACTT-3′) and MyTaq™ HS Red Mix (Bioline).

Commercially used *Lactobacillus plantarum* was obtained from the Food and Nutrition Culture Collection in Universitas Gadjah Mada. For growing isolates and *L. plantarum* in SMB medium and MRS broth medium, materials included dried SMB, MRS broth medium, agar powder, saline solution, and disposable Petri dishes. During the soymilk fermentation by selected isolates and *L. plantarum*, materials such as MRS broth

medium, agar powder, universal indicator, saline solution, sterile diluent, disposable Petri dishes, phenolphthalein, and 0.1 N NaOH were used.

Methods

Preparation of soymilk and SMB

Soybeans were soaked for 24h to soften them, facilitating subsequent processes. After soaking, the soybeans were ground in a solution at a ratio of 8–10 parts water to 1 part beans. The resulting mixture was then separated using cheesecloth, with the liquid portion pasteurized and stored at 4°C. The solid portion, known as SMB, was dried in an oven at 100°C, being stirred every 15 min until a constant weight was achieved (Chun et al. 2007). The dried SMB was subsequently ground and sieved to achieve a consistent particle size (Malik et al. 2022), and finally stored in a cool, dry place until required.

Isolation of LAB from soymilk

Pasteurized soymilk (150 mL) was collected in an airtight container and left to ferment for 3 days. LAB isolation was conducted by serial dilutions of 1 mL of the fermented soymilk mixed with 9 mL of sterile saline solution. From this diluted sample, 0.1 mL was plated on MRS agar using the pour plate method (Sanders 2012). Plates were incubated at 37°C for 24–48 h. Macroscopic differences in colony morphologies were observed, and LAB growth was selected accordingly. Selected colonies were then streaked on MRS agar to obtain pure cultures. Pure cultures were preserved on MRS agar slants and recultured every 2 weeks throughout the research period.

Morphological characterization of isolated LAB

Morphological characterization of isolated LAB was performed via Gram staining as described by Tripathi and Sapra (2023), the spore formation test via the Schaeffer Fulton method as shown by Oktari et al. (2017), and the motility test from Rahayu and Setiadi (2023).

Physiological and biochemical characterization of isolated LAB

Physiological and biochemical characterization of isolated LAB was performed through the catalase test, temperature tolerance test at 4°C, 15°C, 37°C, and 45°C, and salt tolerance test at concentrations of 0%, 4%, and 6.5% NaCl as previously described (Ismail et al. 2018; Yudianti et al. 2020). The difference in optical density (OD) at 600 nm was observed after 48 h of incubation during the temperature and salt tolerance tests.

Genomic DNA extraction and identification by 16S rRNA

Before the extraction of genomic DNA from the isolates, selected isolates were subcultured in MRS broth at 37°C for 24 h. The selection of isolates for sequencing was based on the results of the morphological, physiological, and biochemical characterizations where isolates that showed the characteristics of LAB were selected.

Genomic DNA extraction was performed by using the Bacteria DNA Isolation Kit by Geneaid (Taiwan), according to the manufacturer's instructions. The extracted genomic DNA was assessed via electrophoresis with a 0.8% agarose gel. The resulting genomic DNA was amplified by universal forward and reverse primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1427R (5′-GGTTACCTTGTTACGACTT-3′) via PCR with an initial denaturation at 96°C for 4 min, denaturation at 94°C for 1 min, annealing at 52°C for 1 min and 30 s, extension at 68°C for 8 min, final extension at 68°C for 10 min, and cooling to 12°C for 10 min. Agarose gel electrophoresis

(1.8%) was then performed with 1 × TBE at 100 V with FloroSafe DNA Stain (1st BASE). A 1 Kb Plus DNA ladder was added, where the target molecular weight for the PCR products was 1,500 bp. PCR products were sent to Laboratorium Penelitian dan Pengujian Terpadu in Yogyakarta for sequencing of the 16S rRNA gene. Generated sequences were aligned using BioEdit software and were subsequently analyzed by using the BLAST application for analysis of the NCBI gene bank database.

Growth of isolates and *L. plantarum* in SMB and MRS media

SMB medium was prepared by adding the appropriate amount of dried SMB into double-distilled water, into which 2% (w/v) or 3% (w/v) of SMB were added. MRS broth was prepared according to the manufacturer's instructions. All media was autoclaved at 121°C with a pressure of 1.02 kg/cm^3 for 20 min.

Selected isolates and commercially bought *L. platantarum* were subcultured in MRS broth for 24 h at 37°C. Isolates were transferred to 24 mL of MRS broth and incubated in a shaking incubator until the OD reached 0.2 at a wavelength of 600 nm. Isolates (5%) from the previous media were then transferred into 30 mL of MRS broth. The culture was then incubated with shaking at 37°C until the OD reached 0.9 at 600 nm, which was equivalent to a viable cell population of approximately 10^7 colony-forming units (CFU)/mL (Chun et al. 2007). Isolates (5%) were added to the prepared medium, which included 2% SMB, 3% SMB, and MRS broth for comparison. All cultures were incubated with shaking at 37°C for 8 h. The number of viable cells was counted by performing a serial dilution with sterile saline solution, in which 1 mL was serially diluted in 9 mL of sterile saline solution that was then plated onto MRS agar via the spread plate method. Plates were incubated at 37°C for 48 h. A total plate count was performed, where plates containing between 25–250 colonies were counted and multiplied by the dilution factor to obtain the number of CFU/mL (Parseelan et al. 2019).

Soymilk fermentation by selected isolates and *L. plantarum*

The selected isolate and commercially bought *L. plantarum* from the Food and Nutrition Culture Collection in Universitas Gadjah Mada were recultured in MRS broth at 37°C for 24 h. A sample (1 mL) of the isolate and the commercially bought *L. plantarum* were transferred to 24 mL of MRS broth and was incubated in a shaking incubator until the OD reached 0.2 at a wavelength of 600 nm, which was then transferred into 30 mL MRS broth. Cultures were incubated with shaking at 37°C until the OD reached 0.9 at 600 nm (10^7 CFU/mL). The cultures were then centrifuged at 5,000 rpm for 15 min. Pelleted cells were washed twice with sterile diluent and resuspended and then used as the inoculum for soymilk fermentation. The inoculated soymilk was left to ferment for 6 h, and the pH of the fermentation was measured with a universal indicator every 2 h. The number of viable cells was analyzed through the plate count method with MRS agar, with serial dilutions in sterile saline solution. Plates were incubated at 37°C for 48 h. The total plate count was determined by counting the plates that had between 25–250 colonies and was multiplied by the dilution factor to obtain the number of CFU/mL (Parseelan et al. 2019). The total titratable acidity was determined by adding two drops of phenolphthalein indicator to 9 mL of the soymilk sample and then titrating with 0.1 N NaOH until a pink color was obtained.

Data analysis

Data were analyzed by using IBM SPSS Statistics 27. Means were analyzed by using the t-test with a significance level of $P < 0.05$. Furthermore, the method demonstrated by (Gunawan et al. 2021) was used to determine the total lactic acid percentage.

RESULTS AND DISCUSSION

Morphological characterization of isolated LAB

The morphological characterization of LAB was performed through macroscopic and microscopic observation of colonies. Fourteen isolates were initially obtained as described in Table 1.

The main differences between the isolates were observed from the colony shape, where isolates 1, 3, 4, 9, 10, 11, 12, and 14 had a circular colony shape, whereas isolates 2, 5, 6, 7, 8, and 13 had a punctiform colony shape. Furthermore, when observed macroscopically from the other parameters, all the colonies were quite similar and exhibited a colony arrangement of coccobacillus, were white in color, had a convex colony elevation, an entire colony margin, and a smooth colony surface. Thus, all isolates that were obtained had characteristics of LAB, whose colonies are either circular or punctiform in shape, with a colony arrangement of coccus, bacillus, or coccobacillus, white to yellow in color, with a convex colony elevation, entire colony margin, and smooth colony surface (Elbanna et al. 2018; Bayu et al. 2023; Rahayu & Setiadi 2023).

During the microscopic observation of the isolates, Gram staining and spore formation testing were performed, to visualize isolated cell shapes. Gram staining includes the performance of a staining process where four different reagents (crystal violet, iodine, 70% ethanol, and, safranin) are used to determine whether a bacterium is gram-positive or negative based on the thickness of the peptidoglycan layer in the bacterial cell walls (Rahayu & Setiadi 2023). The staining process works by adding crystal violet as the primary stain, followed by the addition of iodine, which causes the crystal violet stain to remain in the peptidoglycan layer of the bacterial cell wall. This is followed by the addition of 70% ethanol, which washes the stain from the peptidoglycan (Tripathi & Sapra 2023). Lastly, safranin is added as a counterstain to color the viable cells in the microscope slide. Through this process, gram-positive bacteria are stained purple because of the presence of a thick peptidoglycan layer,

Table 1. Morphological characterization of isolated LAB.

Isolate Code	Shape	Arrangement	Color	Elevation	Margin	Surface	Gram	Spore	Motility
1	Circular	Coccobacillus	White	Convex	Entire	Smooth	+	−	+
2	Punctiform	Coccobacillus	White	Convex	Entire	Smooth	+	−	−
3	Circular	Coccobacillus	White	Convex	Entire	Smooth	+	−	+
4	Circular	Coccobacillus	White	Convex	Entire	Smooth	+	−	−
5	Punctiform	Coccobacillus	White	Convex	Entire	Smooth	+	−	−
6	Punctiform	Coccobacillus	White	Convex	Entire	Smooth	+	−	+
7	Punctiform	Coccobacillus	White	Convex	Entire	Smooth	+	−	+
8	Punctiform	Coccobacillus	White	Convex	Entire	Smooth	+	−	−
9	Circular	Coccobacillus	White	Convex	Entire	Smooth	+	−	−
10	Circular	Coccobacillus	White	Convex	Entire	Smooth	+	−	+
11	Circular	Coccobacillus	White	Convex	Entire	Smooth	+	−	+
12	Circular	Coccobacillus	White	Convex	Entire	Smooth	+	−	+
13	Punctiform	Coccobacillus	White	Convex	Entire	Smooth	+	−	+
14	Circular	Coccobacillus	White	Convex	Entire	Smooth	+	−	+

whereas gram-negative bacteria are stained red as the peptidoglycan layer is thinner when compared with that of gram-positive bacteria (Tripathi & Sapra 2023).

The spore formation assay was performed using the Schaeffer Fulton method that uses malachite green stain and steam to enable the stain to enter the cells, penetrating the cell walls and the spores; safranin is used as a counterstain to stain the viable cells (Oktari et al. 2017). In this assay, spores are stained green while the viable cells are stained a red-pink color (Oktari et al. 2017).

All isolates had a negative result during the spore formation test (Table 1), indicating that the isolates did not contain spores, which agrees with the literature as LAB do not form spores (Rahayu & Setiadi 2023).

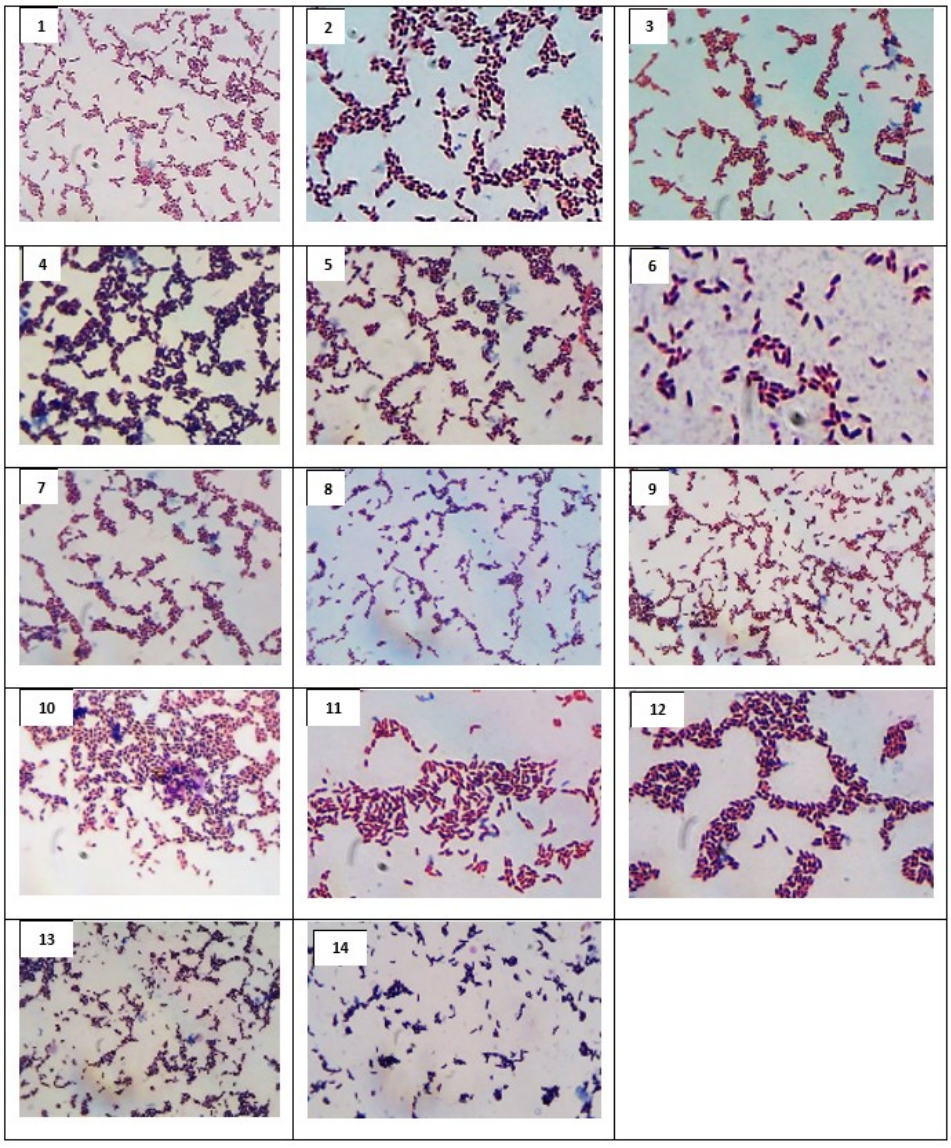

Figure 1. Colony morphology of isolated LAB as a result of Gram staining observed through a microscope with 1000× magnification.

The gram staining results are shown in Figure 1, which shows the colonies of the isolates after Gram staining with 1000× magnification. All isolates exhibited a purple stain, indicating that they were all gram positive bacteria. This result agrees with the literature that states that all LAB are gram positive bacteria (Bayu et al. 2023; Rahayu & Setiadi 2023).

The motility test determines if a bacterial isolate is motile or

nonmotile through the inoculation of the bacteria into a semisolid medium (Rahayu & Setiadi 2023). A positive result in the motility test is indicated by turbidity in the inoculated medium while a negative result is shown by the growth of the isolate along the streak line. The results of the motility test in Table 1 show that most isolates (1, 3, 6, 7, 10, 11, 12, 13, and 14) were motile. Generally, LAB are nonmotile as these lack structures such as flagella for motility (Rahayu & Setiadi 2023). Therefore, isolates 2, 4, 5, 8, and 9 that showed a negative result from the motility test were selected as potential LAB isolates while the rest of the isolates that showed a positive result were discarded.

Physiological and biochemical characterization of isolated LAB

The physiological and biochemical characterization of the isolated LAB included temperature tolerance, salt tolerance, and catalase tests to confirm the selected isolates as LAB.

The catalase test was performed to determine if the selected isolates could produce catalase, which changes hydrogen peroxide (H_2O_2) into water and oxygen (Ismail et al. 2018). A positive result of the catalase test is indicated by the production of bubbles when a drop of 3% H_2O_2 is added to the bacterial isolate, indicating the formation of oxygen gas, whereas a negative result is indicated by the absence of the formation of oxygen gas bubbles (Ismail et al. 2018). All isolates showed a negative result for the catalase test (Table 2), which agrees with the theory that indicates that LAB do not produce catalase (Ismail et al. 2018). This is because LAB are facultative anaerobic organisms that prefer anaerobic conditions to grow but can tolerate the presence of oxygen, unlike aerobic organisms that require the presence of oxygen to grow; therefore, H_2O_2 is created as a product of LAB metabolism and can be toxic, requiring catalase to breakdown the H_2O_2 into oxygen and water (Ismail et al. 2018).

Table 2. Physiological and biochemical characterization of isolated LAB.

Isolate Code	Temperature Tolerance (°C)				Catalase
	4	15	37	45	
2	+[a]	+	+	−	−[c]
4	−[b]	−	+	−	−
5	−	+	+	+	−
8	−	+	+	+	−
9	−	+	+	+	−

Note: a: Growth of isolate; b: death of isolate; c: negative reaction

The temperature tolerance test was performed to determine the growth ability of the isolates at different temperatures (4°C, 15°C, 37°C, and 45°C) over 48 h. LAB are generally known as mesophilic bacteria, with an optimum growth temperature from 25°C to 37°C (Bayu et al. 2023). However, some LAB can also grow and survive at temperatures lower or higher than the optimum temperature. Table 2 shows the ability of the isolates to grow at temperatures of 4°C, 15°C, 37°C, and 45°C, where the (+) symbol indicates the ability of the isolate to grow in the corresponding temperature while the (–) symbol indicates the inability of the isolate to grow in the corresponding temperature, which was determined by the obtaining the OD at 600 nm of the isolate at 0 and 48 h. Table 2 shows that all isolates could grow at 37°C, which confirms that they could potentially be LAB because of the mesophilic characteristic as described by Bayu et al. (2023). Furthermore, only isolate 2 could grow at 4°C while most of the isolates could grow at 15°C and 45°C.

Figure 2 shows the increase in the OD during the temperature tolerance test of selected isolates, which shows a trend where all isolates had an optimum growth temperature of 37°C. Furthermore, all isolates could grow well at 45°C, although not all the results exhibited a significant difference (Table 2). Testing for temperature tolerance is important for LAB, not only for the screening process but also to determine any probiotic potential of the isolated LAB. During the milk fermentation process, the milk is initially pasteurized, which requires high temperatures, and a temperature of 37°C is usually used for the fermentation process. Therefore, when selecting LAB for the fermentation process, not all LAB have the ability to survive high temperatures used for pasteurization (Ayo-Omogie & Okorie 2016).

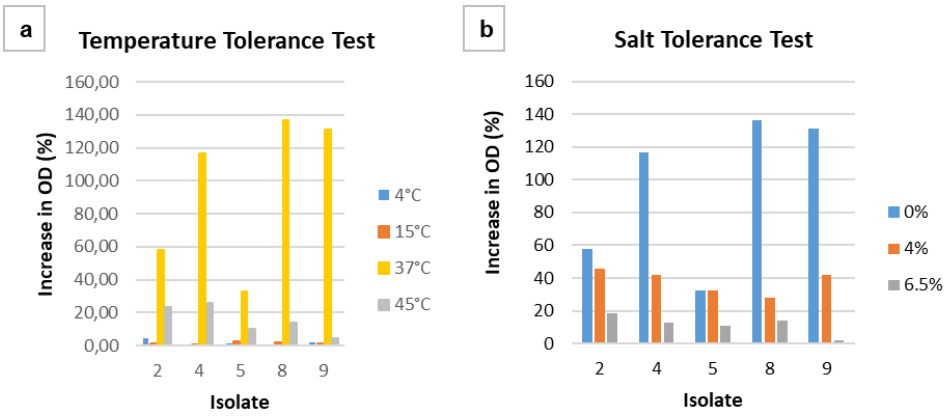

Figure 2. Increase in optical density (OD) during the (a) temperature tolerance test and (b) salt tolerance test of the isolated LAB.

The salt tolerance test determines the growth ability of selected isolates in different concentrations of salt, specifically at concentrations of 0%, 4%, and 6.5%, which is shown by Figure 2. This test was performed because some LAB are considered moderately halophilic bacteria, which means that they can withstand salt concentrations between 3% and 15% (Ismail et al. 2018; Karyantina et al. 2020). The salt tolerance test was conducted by inoculating the selected isolates into mediums with different salt concentrations (0%, 4%, and 6.5%) and comparing the OD at 0 h and after a 48-h incubation. Most of the isolated LAB thrived at 0% salt while 4% salt yielded a higher OD than that at 6.5%. This means that most of the bacteria could survive better at a salt concentration of 4% than at a salt concentration of 6.5%. The results obtained were in accordance with the theory stated by (Karyantina et al. 2020), where most of the isolated LAB can survive at salt concentrations from 2% to 6%, whereas a higher salt concentration resulted in a negative result, categorizing LAB as moderate halophiles. This test indicates that LAB have the potential to be used as a probiotic during the fermentation of salty food and beverages.

Genomic DNA extraction and identification by 16S rRNA sequencing

Genomic DNA was extracted from isolates 4 and 8 because of their supporting characteristics for use as a probiotic. Isolates 4 and 8 showed positive results during the temperature tolerance test at 45°C and showed positive results in tolerating different concentrations of salt during the salt tolerance test, which supports their potential for use as probiotic bacteria. The high-temperature tolerance indicates that the isolate can be used to create probiotic products as fermentation usually requires

an optimum temperature of 37°C (Ayo-Omogie & Okorie 2016). Furthermore, during the consumption of probiotic products, the temperature of the human gut can typically reach 37°C, which could affect the desired effect of the probiotic product if the probiotic bacteria lose viability before providing the effect for the consumer (Ayo-Omogie & Okorie 2016). A high salt tolerance is required for probiotic bacteria to ensure that the bacteria can survive the high salt condition created by the presence of bile salt in the body, where a lack of viability caused by the presence of salt prevents the delivery of the expected effect of the probiotic product (Ayo-Omogie & Okorie 2016).

Gel electrophoresis showed that the genomic DNA was successfully isolated from both isolates 4 and 8 (Figure 3). The DNA was then amplified as described in the Methods section.

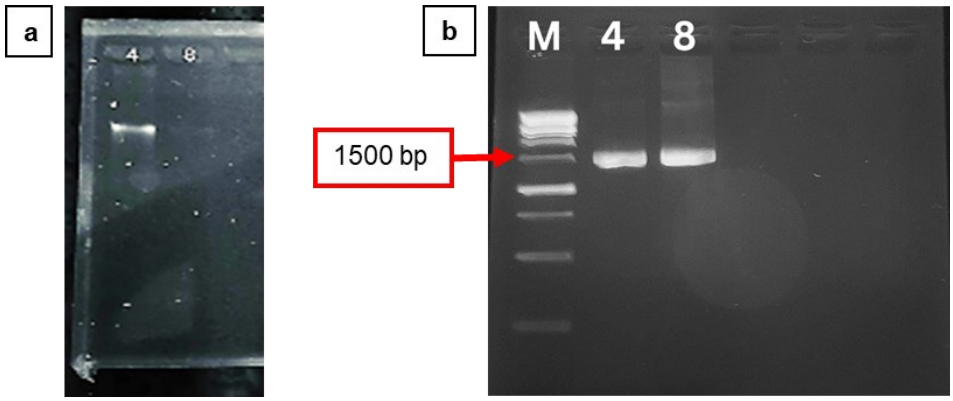

Figure 3. Electrophoresis result for the (a) DNA isolation of isolates 4 and 8 and (b) PCR product when amplified with 16S rRNA universal primers with 1 Kb plus marker (M).

Figure 3 shows the gel electrophoresis of the amplified PCR product from DNA extracted from isolates 4 and 8. A single band at 1,500 bp indicates that the DNA was successfully amplified with the primers. The PCR product was sequenced, and the sequencing results aligned to create a consensus sequence, which was then analyzed via BLAST to compare with sequences of species available in the NCBI GenBank database. From the BLAST results, isolate number 4 was identified as *Weisella confusa* strain NWAFU 8,001, with a percent identity of 99.71% and a query cover of 100% while isolate 8 was identified as *Weissella confusa* strain 3,273 with a percent identity of 100% and a query cover of 100%.

W. confusa is described as a gram-positive bacteria with a coccus colony shape that is facultatively anaerobic, nonmotile, and catalase-negative, has negative spore formation, and is considered a LAB (Fessard & Remize 2017; Spiegelhauer et al. 2020). Before recent classification, *W. confusa* used to be called *Lactobacillus confusus*; however, the *Weissella* genus was created in 1993 following 16S rRNA sequencing, and *L. confusus* was changed to *W. confusa* (Spiegelhauer et al. 2020). Macroscopically, *W. confusa* colonies can be observed as rods in pairs or chains. Physiologically, *W. confusa* is considered a facultative anaerobic bacteria, which means that *W. confusa* can live in either aerobic or anaerobic conditions and metabolize with or without the presence of oxygen (Fessard & Remize 2017). Fermentation occurs in the absence of oxygen, where lactic acid, carbon dioxide, ethanol, and acetic acid are produced as a final result of the fermentation process. *W. confusa* can grow in temperatures between 15°C to 37°C, and several strains can grow in temperatures up to 47°C (Fessard & Remize 2017). These results fully correspond to the morphological and physiological characteristics obtained for isolates 4 and 8.

W. confusa has been reportedly isolated from various sources, including fermented foods, such as fermented fruits, vegetables, dairy and meat products, indicating that the species can adjust to different growth conditions in different environments (Fessard & Remize 2017). Examples of reports of *W. confusa* isolated from fruit and vegetable sources include during the fermentation of Tuaw jaew, which is a type of fermented soybeans from Thailand, and during the fermentation of Korean leek (*Allium tuberosum* Rottler) (Yang et al. 2014). *W. confusa* has also been isolated from dairy products, including from Nono, a fermented skimmed milk from Nigeria, and from the surface of an Italian cheese (Di Cagno et al. 2007; Ayeni et al. 2011). Furthermore, *W. confusa* has been identified from clinical cases in humans, such as from the skin, gastrointestinal tract, and blood, where the disease-causing ability is still questioned (Fairfax et al. 2014; Spiegelhauer et al. 2020).

As *W. confusa* has been isolated from many fruit, vegetable, and dairy sources, the probiotic ability in fermented products has been explored. While most reports on the isolation of *W. confusa* were conducted using spontaneous fermentation, the use of *W. confusa* as a starter culture is being explored, especially as *W. confusa* can ferment many different types of food and beverages, supporting its ability to survive in different types of conditions. This is because when spontaneous fermentation occurs, it is challenging to control the microorganisms that grow in the product as this type of fermentation usually contains many different types of microorganisms, resulting in a product that can have different characteristics from one batch to another (Fessard & Remize 2017). However, when starters are used, the fermentation usually only contains one or a few types of microorganisms of known fermentation ability in suitable amounts, resulting in a product that has uniform characteristics (Fessard & Remize 2017).

Growth of isolates and *L. plantarum* in SMB and MRS media

The growth of isolates and *L. plantarum* in SMB and MRS media was conducted to compare the growth of isolates 4 and 8, and commercially bought *L. plantarum* in SMB as an alternative growth medium compared with their growth in the commercially available MRS medium. Generally, alternative mediums used as the standard or commercial medium can be expensive while alternative media are usually more low cost as the materials used are mainly from locally available food sources (Hayek et al. 2019; Malik et al. 2022).

Two different concentrations of SMB (2% and 3%) were used in this study, as pictureed in Figure 4. Visually, the SMB alternative medium appeared white in color while the SMB medium with a concentration of 3% had an opaque color. However, fully dissolving the SMB in the distilled water was difficult because of the high fiber content of soybeans; therefore, the SMB medium may be more appropriately used as a solid/agar medium than as a liquid/broth medium as the opaque color makes it difficult to observe bacterial growth in liquid culture.

To perform the growth comparison between the alternative SMB and standard MRS media, a similar number of viable bacterial cells were inoculated into the same amount of medium. The media was then incubated with shaking for 8 h, and the final CFU/mL of the media was calculated through serial dilution and the total plate count.

Figure 4c demonstrates that a similar trend was present in all isolates in the different types of media used. The highest CFU/mL count was observed in the MRS broth medium for all isolates, followed by 2% SMB, and then 3% SMB. The highest CFU/mL count was observed with

isolate 8 (*W. confusa*) in all media used when compared with the other isolates; *L. plantarum* has the second highest CFU/mL count followed by isolate 4 (also identified as *W. confusa*).

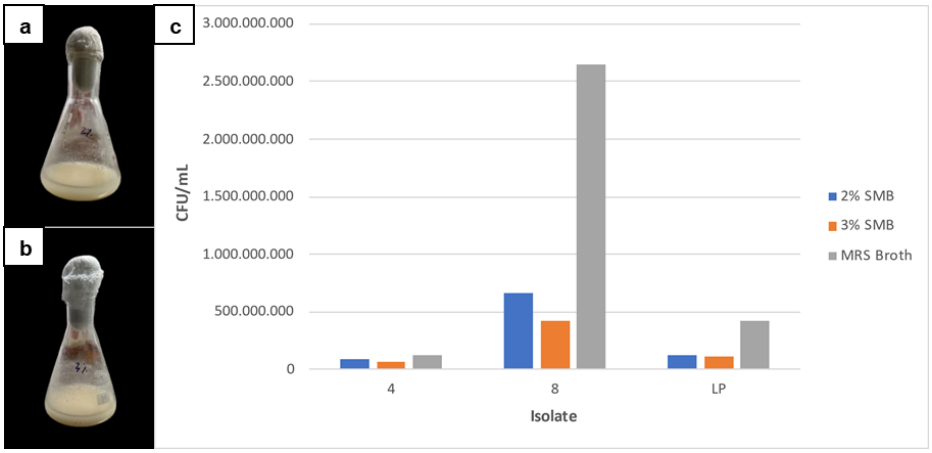

Figure 4. 2% (a) and 3% (b) SMB medium created to be inoculated with the selected isolates and *L. plantarum*; (c) Growth comparison through CFU/mL of isolates 4 and 8, and *L. plantarum* (LP) in 2% and 3% SMB (Okara) and MRS broth media after 8 h of incubation.

The CFU/mL count was highest with the use of MRS broth as this commercially made medium is considered a selective medium for LAB. This is because MRS contains all of the essential micro and micronutrients that are required for the growth of LAB and has been used as a standard medium for their cultivation for approximately 63 years (Hayek et al. 2019). Typical MRS medium usually contains high amounts of peptone that serves as a source of nitrogen content in the medium and can maintain an ideal pH level for LAB growth. However, a major limitation of the use of MRS medium is the expensive price, which can be an issue for research conducted in developing countries.

When compared between the different concentrations of the alternative SMB medium, between all the isolates, the 2% SMB medium had a higher CFU/mL count than that of the 3% SMB medium, although the differences between in the CFU/mL count between these media were not significant. First, soy is considered to contain high amounts of protein, which could serve as a nitrogen source for the growth of LAB as a high nitrogen content is required for its growth, supporting the use of soybeans or SMB for the use as an alternative media source. The differences between the results of the CFU/mL counts in the 2% and 3% SMB could be due to the higher water content that is available in the 2% SMB medium compared with that in the 3% SMB medium, which enables the nutrients that are available in the SMB to be more easily dissolved in the 2% medium, making the nutrients more readily available for the LAB to use (Alp & Bulantekin 2021).

Unfortunately, limited research has explored the use of SMB as a source of alternative medium, especially for LAB. Similar research used dried soybeans to make soybean flour as an alternative medium for the cultivation of microorganisms such as *Bacillus cereus* and evaluated different concentrations of soy flour along with different particle sizes (Utomo et al. 2022). A higher soy flour concentration with a smaller particle size was shown to increase the amount of *Bacillus* bacteria grown.

Soymilk fermentation with isolate 8 and *L. plantarum*

Soymilk fermentation was conducted by inoculating a similar number of viable cells from isolate 8 (*W. confusa*) and *L. plantarum* to compare their

fermentation ability and to determine the probiotic ability of the isolated bacteria when compared with a commercially available LAB.

The highest CFU/mL count was achieved with isolate 8 (5.5×10^9 CFU/mL) when compared with that of *L. plantarum* (3.27×10^9 CFU/mL) as shown by Figure 5. The higher CFU/mL count obtained with isolate 8 is because the isolate was isolated from soymilk, which means that the isolate may be adapted to the growth conditions in soymilk and could support a higher CFU/mL while the commercially bought *L. plantarum* was not fully adapted to these growth conditions. However, similar research conducted by Chun et al. (2007), which also used *W. confusa* and *L. plantarum* for the fermentation of soymilk for 9–12 h produced different results, where *L. plantarum* achieved a higher CFU/mL count than *W. confusa*. This difference may be caused by the difference in the strains of *Weissella* bacteria used. In this study, the *W. confusa* strain (isolate 8) was obtained by isolating the bacteria from soymilk and was better adapted to the growth conditions.

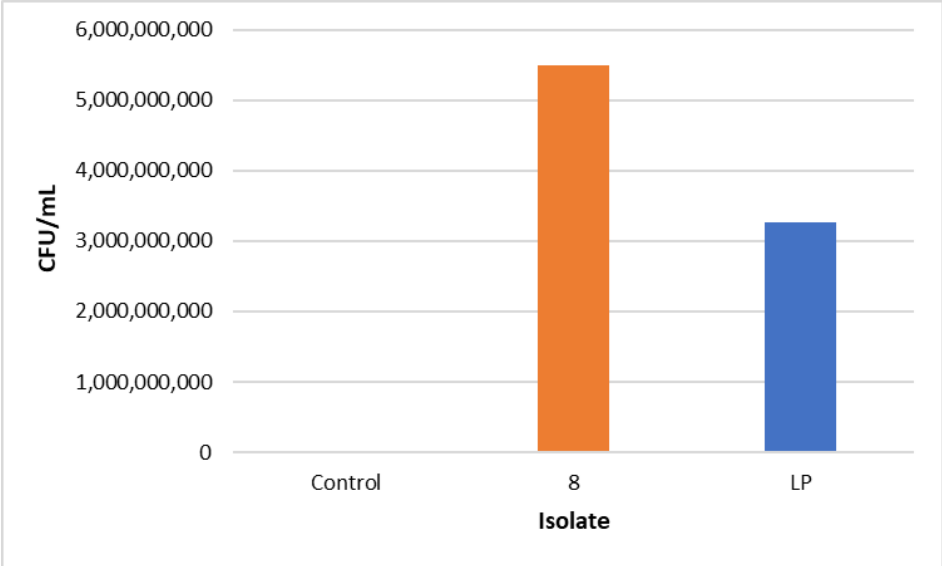

Figure 5. Comparison of CFU/mL of soymilk inoculated with 5% of isolate 8 and *L. plantarum* (LP) for 6 h at 37°C.

Furthermore, the results of the fermentation of soymilk with the isolated bacteria (isolate 8) showed that this isolate could successfully create a probiotic product as the final CFU/mL count was $>10^7$ CFU/mL, which is considered the minimum amount of bacteria required in probiotic products to provide a positive effect to the host (Teh et al. 2010).

During the soymilk fermentation process, the pH of the soymilk was determined by using a universal indicator every 2 h to understand the change in pH throughout the fermentation process, as shown by Figure 6. A drop in pH is usually a sign of the formation of lactic acid as a result of the metabolism by LAB present in the soymilk, showing that a continuous fermentation process is being performed (Liu et al. 2016). The largest drop in pH was produced in the isolate 8 culture (Figure 6a), where the pH of the milk decreased after 2 h of fermentation when compared with that in the *L. plantarum* and control cultures. The difference in the drop in pH between cultures of isolate 8 and *L. plantarum* could be due to the difference in the speed of the fermentation process of both isolates, where isolate 8 could be more well adapted to the growth conditions and has the ability to better ferment the soymilk than the commercially bought *L. plantarum* and produce more lactic acid to lower the pH

of the soymilk. A similar result was observed in a previous study (Chun et al. 2007), where the fermentation of soymilk with various strains of LAB, including *W. confusa* and *L. plantarum* reduced the pH to between 4.4. and 4.6 because of lactic acid production at 6 h of fermentation.

Titratable acidity is used to calculate the total lactic acid content of the fermented soymilk (Tyl & Sadler 2017; Gunawan et al. 2021). Figure 6b shows that the highest titratable acidity was achieved by isolate 8, followed by *L. plantarum*, and lastly by the control. This result corresponds to the final pH of the soymilk fermentation process, where the lowest pH was achieved in the same order. According to Chun et al. (2007), high titratable acidity is commonly achieved by strains that produce a lower pH during the fermentation process as a result of a higher acidity content. This result is reflected here as isolate 8 achieved the lowest pH while also achieving a higher titratable acidity.

Furthermore, the total lactic acid percentage (Figure 6c) was calculated as reported by Gunawan et al. (2021), which showed that the highest lactic acid percentage was achieved by isolate 8, followed by that achieved by the commercially bought *L. plantarum* and lastly by the control. The difference in the value of the total lactic acid percentage can be an indicator of the flavor that is created by the different isolates during soymilk fermentation. Furthermore, the higher the lactic acid content, the more difficult it is for the soymilk to spoil as the pH is lower, which therefore discourages the growth of spoilage bacteria in the product (Gunawan et al. 2021).

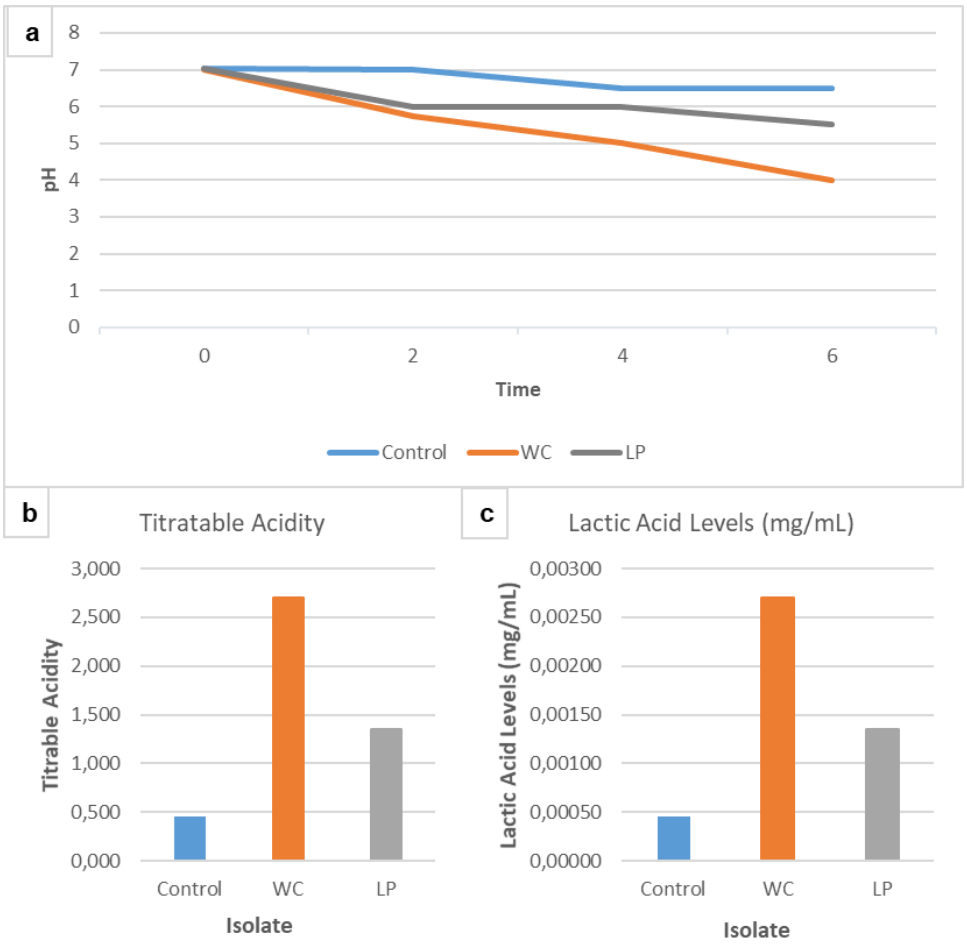

Figure 6. (a) pH of the fermented soymilk inoculated with 5% of isolate 8 and *L. plantarum* (LP) for 6 h at 37°C; (b) Titratable acidity and (c) total lactic acid content of soymilk fermented with isolate 8 and *L. plantarum* (LP).

CONCLUSIONS

This study showed that the isolates selected from fermented soymilk were both identified as *W. confusa* via sequencing of the 16S rRNA gene. *W. confusa* is considered an LAB that has been commonly isolated from many different sources, even forming spontaneous fermentations in foods and beverages. SMB could be used to culture LAB. The SMB was first dried until a constant weight was achieved, followed by grinding and sifting to obtain a uniform particle size. A concentration of 2% SMB was more effective in producing higher numbers of viable LAB colonies than that of 3%. LAB cultured from SMB can ferment soymilk, where the probiotic ability was demonstrated with a final CFU/mL count of 5.5×10^9 CFU/mL, which was higher than that of the commercially bought LAB isolate and reached the minimum standard of a probiotic product.

AUTHOR CONTRIBUTION

F.D.R. and Y.A.P. designed the research and supervised the research process. F.D.R. and Y.S. collected and analyzed the data. F.D.R wrote the manuscript.

ACKNOWLEDGMENTS

We would like to express our sincere gratitude to Tri Purwanti, as the laboratory technician, for her invaluable assistance throughout the study. We also extend our heartfelt thanks to our laboratory partners for their unwavering moral support, which has been instrumental in the completion of this research. Lastly, we are deeply grateful to the editors of this journal for their guidance and support throughout the publishing process.

CONFLICT OF INTEREST

The authors do not have any conflicts of interest during the research.

REFERENCES

Alp, D. & Bulantekin, Ö., 2021. The microbiological quality of various foods dried by applying different drying methods: a review. *European Food Research and Technology*, 247(6), pp.1333–1343. doi: 10.1007/s00217-021-03731-z.

Aritonang, S.N. et al., 2017. Isolation and Identification of Lactic Acid Bacteria from Okara and Evaluation of Their Potential as Candidate Probiotics. *Pakistan Journal of Nutrition*, 16(8), pp.618–628. doi: 10.3923/pjn.2017.618.628.

Aydar, E.F., Tutuncu, S. & Ozcelik, B., 2020. Plant-based milk substitutes: Bioactive compounds, conventional and novel processes, bioavailability studies, and health effects. *Journal of Functional Foods*, 70, 103975. doi: 10.1016/j.jff.2020.103975.

Ayeni, F.A. et al., 2011. Evaluation of the functional potential of Weissella and Lactobacillus isolates obtained from Nigerian traditional fermented foods and cow's intestine. *International Journal of Food Microbiology*, 147(2), pp.97–104. doi: 10.1016/j.ijfoodmicro.2011.03.014.

Ayo-Omogie, H. & Okorie, E., 2016. In vitro Probiotic Potential of Autochthonous Lactic Acid Bacteria and Microbiology of Kunu Made from Mixed Grains. *British Microbiology Research Journal*, 14(4), pp.1–10. doi: 10.9734/BMRJ/2016/25403.

Bayu, H.H. et al., 2023. Isolation and Identification of Lactic Acid Bacteria from *Channa* sp. as Potential Probiotic. *Jurnal Pembelajaran dan Biologi Nukleus*, 9(1), pp.75–84. doi: 10.36987/jpbn.v9i1.3551.

Chun, J. et al., 2007. Conversion of Isoflavone Glucosides to Aglycones in Soymilk by Fermentation with Lactic Acid Bacteria. *Journal of Food Science*, 72(2). doi: 10.1111/j.1750-3841.2007.00276.x.

Davy, P. & Vuong, Q.V., 2022. Soy Milk By-product: Its Composition and Utilisation. *Food Reviews International*, 38(sup1), pp.147–169. doi: 10.1080/87559129.2020.1855191.

Di Cagno, R. et al., 2007. Characterization of Italian Cheeses Ripened Under Nonconventional Conditions. *Journal of Dairy Science*, 90(6), pp.2689–2704. doi: 10.3168/jds.2006-654.

Elbanna, K. et al., 2018. In vitro and in vivo evidences for innate immune stimulators lactic acid bacterial starters isolated from fermented camel dairy products. *Scientific Reports*, 8(1), 12553. doi: 10.1038/s41598-018-31006-3.

Fairfax, M.R., Lephart, P.R. & Salimnia, H., 2014. Weissella confusa: problems with identification of an opportunistic pathogen that has been found in fermented foods and proposed as a probiotic. *Frontiers in Microbiology*, 5. doi: 10.3389/fmicb.2014.00254.

Fessard, A. & Remize, F., 2017. Why Are Weissella spp. Not Used as Commercial Starter Cultures for Food Fermentation? *Fermentation*, 3(3), 38. doi: 10.3390/fermentation3030038.

Goda, H., Barakat, O. & Ali, M., 2011. Potential Exploitation of Okara as an Alternative Medium for Edible Pleurotus ostreatus Mycelium Production. *Arab Journal of Biotechnology*, 14, pp.139–153.

Gunawan, S. et al., 2021. Reaction kinetics of lactic acid fermentation from bitter cassava (Manihot glaziovii) starch by Lactobacillus casei. *Indonesian Journal of Biotechnology*, 26(1), 7. doi: 10.22146/ijbiotech.54119.

Hayek, S.A. et al., 2019. Cultivation media for lactic acid bacteria used in dairy products. *Journal of Dairy Research*, 86(4), pp.490–502. doi: 10.1017/S002202991900075X.

Ismail, Y., Yulvizar, C. & Mazhitov, B., 2018. Characterization of lactic acid bacteria from local cow´s milk kefir. *IOP Conference Series: Earth and Environmental Science*, 130, 012019. doi: 10.1088/1755-1315/130/1/012019.

Karyantina, M. et al., 2020. Moderate Halophilic Lactic Acid Bacteria from *Jambal roti*: A Traditional Fermented Fish of Central Java, Indonesia. *Journal of Aquatic Food Product Technology*, 29(10), pp.990–1000. doi: 10.1080/10498850.2020.1827112.

Kim, J., 2019. *Transforming okara into a microalgae culture medium.* Nanyang Technological University. doi: 10.32657/10220/49477.

Liu, Y. et al., 2016. Characterization of Lactobacillus pentosus as a starter culture for the fermentation of edible oyster mushrooms (Pleurotus spp.). *LWT - Food Science and Technology*, 68, pp.21–26. doi: 10.1016/j.lwt.2015.12.008.

Malik, N.H.A., Simarani, K. & Aziz, M.A., 2022. Soybean as an Alternative Nutrient Medium for *Bacillus subtilis* Growth. *Malaysian Applied Biology*, 51(4), pp.67–74. doi: 10.55230/mabjournal.v51i4.12.

Malik, T.F. & Panuganti, K.K., 2022. Lactose Intolerance. In *StatPearls*. Treasure Island (FL): StatPearls Publishing. http://www.ncbi.nlm.nih.gov/books/NBK532285/

Oktari, A. et al., 2017. The Bacterial Endospore Stain on Schaeffer Fulton using Variation of Methylene Blue Solution. *Journal of Physics: Conference Series*, 812, 012066. doi: 10.1088/1742-6596/812/1/012066.

Parseelan, A. et al., 2019. Aerobic Plate Count of Milk and Dairy Products Marketed in Different Zones of Chennai. *International Journal of Livestock Research*, 9(3). doi: 10.5455/ijlr.20180711104721.

Rahayu, H.M. & Setiadi, A.E., 2023. Isolation and Characterization of Indigenous Lactic Acid Bacteria from Pakatikng Rape, Dayak's Traditional Fermented Food. *Jurnal Penelitian Pendidikan IPA*, 9(2), pp.920–925. doi: 10.29303/jppipa.v9i2.2801.

Rizzo, G. & Baroni, L., 2018. Soy, Soy Foods and Their Role in Vegetarian Diets. *Nutrients*, 10(1), 43. doi: 10.3390/nu10010043.

Sanders, E.R., 2012. Aseptic Laboratory Techniques: Plating Methods. *Journal of Visualized Experiments*, (63), 3064. doi: 10.3791/3064.

Spiegelhauer, M.R. et al., 2020. A case report of polymicrobial bacteremia with Weissella confusa and comparison of previous treatment for successful recovery with a review of the literature. *Access Microbiology*, 2(5). doi: 10.1099/acmi.0.000119.

Teh, S. et al., 2010. Enhanced Growth of Lactobacilli in Soymilk upon Immobilization on Agrowastes. *Journal of Food Science*, 75(3). doi: 10.1111/j.1750-3841.2010.01538.x.

Tripathi, N. & Sapra, A., 2023. Gram Staining. In *StatPearls*. Treasure Island (FL): StatPearls Publishing.

Tyl, C. & Sadler, G.D., 2017. pH and Titratable Acidity. In *Food Analysis*. Food Science Text Series. Cham: Springer International Publishing, pp.389–406. doi: 10.1007/978-3-319-45776-5_22.

Utomo, A.S., Harismah, K. & Mulyaningtyas, A., 2022. Making *Bacillus cereus* Bacterial Growth Media from Soybeans (*Glycine max* (L.) Merril): In 4th International Conference Current Breakthrough in Pharmacy (ICB-Pharma 2022). Sukoharjo, Indonesia. doi: 10.2991/978-94-6463-050-3_26.

Vanga, S.K. & Raghavan, V., 2018. How well do plant based alternatives fare nutritionally compared to cow's milk? *Journal of Food Science and Technology*, 55(1), pp.10–20. doi: 10.1007/s13197-017-2915-y.

Yang, J. et al., 2014. Selection of functional lactic acid bacteria as starter cultures for the fermentation of Korean leek (Allium tuberosum Rottler ex Sprengel.). *International Journal of Food Microbiology*, 191, pp.164–171. doi: 10.1016/j.ijfoodmicro.2014.09.016.

Yudianti, N.F. et al., 2020. Isolation and Characterization of Lactic Acid Bacteria from Legume Soaking Water of Tempeh Productions. *Digital Press Life Sciences*, 2, 00003. doi: 10.29037/digitalpress.22328.