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Utilization of *Weissella confusa* isolated from soymilk for soymilk fermentation

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Abstract. Soy milk is widely used as a substitute for dairy milk, mainly due to intolerances towards the lactose content in dairy milk. This type of intolerance is prevalent worldwide as over 65% of people are intolerant to lactose, which could cause effects such as bloating and diarrhea. However, a main disadvantage of soy milk is its beany flavor, which is caused by the enzyme lipoxygenase which breaks down polyunsaturated fatty acids. One of the popular methods of remove this flavor is by the fermentation of soy milk. During this study, the fermentation of soymilk is done by *Weissella confusa*, which belongs to the lactic acid bacteria group, isolated from soy milk and compared with *Lactiplantibacillus plantarum*. The overall fermentation ability is observed through the (1) colony forming units/mL of the bacteria, (2) pH, and (3) lactic acid content, which was done through the calculation of the titratable acidity of the fermented product, after 6 hours of fermentation at 37 °C. The results showed that the *Weissella confusa* isolate obtained an overall better fermentation ability than *Lactiplantibacillus plantarum*, with higher number of CFU/mL, reaching 5.5×10^9 CFU/mL, a lower pH of 4, and a higher lactic acid content of 0.27%. However, further studies are required to verify the use of *Weissella confusa* as a probiotic bacteria for human consumption.

1 Introduction

One of the most popular soy-derived products is soy milk, which is widely used throughout the globe to substitute dairy milk, typically as a result of dietary preferences or intolerances. It has been estimated that around 65% of the global population is intolerant to lactose, which is present in dairy products [1]. Additionally, lactose intolerance affects 75% to 100% of people in East Asia [2].

A significant drawback of soymilk is its beany flavor, which is caused by the lipoxygenase enzyme breaking down polyunsaturated fatty acids [3]. One of the most common methods to remove this flavor is through the fermentation of soymilk by utilizing lactic acid bacteria (LAB), which are known to ferment a variety of products, producing lactic acid during the process. LAB may be probiotic bacteria, as the bacteria have the ability to remain live in the human microflora, providing health benefits for the host during its consumption [4].

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During this study, the fermentation of soymilk is done by *Weissella confusa*, which belongs to the LAB group, previously isolated from soy milk, and compared with commercially available *Lactiplantibacillus plantarum* as a commercial comparison. The fermentation ability of the bacteria is compared through its pH throughout the 6-hour fermentation process, colony forming units (CFU)/mL of the bacteria, and lactic acid content of the end product. The calculation of the CFU/mL of the product also serves as the determination of the product as a probiotic product, with a standardized minimum of 10^6 CFU/mL of viable bacteria to provide a positive benefit to the host [5].

2 Methods

2.1 Genomic identification of the isolated *Weissella confusa* from fermented soymilk by 16S rRNA

The *Weissella confusa* isolate used during this study was previously isolated from soymilk through serial dilution, and plated on MRS agar. Pure cultures were then obtained through morphological differences. Before the genomic DNA of the isolate was extracted, the chosen isolate was subcultured in MRS broth for 24 hours at 37 °C. Bacteria DNA Isolation Kit from Geneaid (Taiwan) was used to extract genomic DNA. The availability of the isolated genomic DNA was confirmed using 0.8% agarose gel electrophoresis. Using universal forward and reverse primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1427R (5'-GGTTACCTTGTTACGACTT-3'), the resultant genomic DNA was amplified using PCR. The program that was used constituted of initial denaturation at 96 °C for 4 minutes, denaturation at 94 °C for 1 minute, annealing at 52 °C for 1 minute and 30 seconds, extension at 68 °C for 8 minutes, final extension at 68 °C for 10 minutes, and cool down at 12 °C for 10 minutes. Gel electrophoresis was done by using 1.8% agarose and 1× Tris Borate-EDTA buffer (TBE) at a voltage of 100 V with FloroSafe DNA Stain (1st BASE). DNA ladder (1 Kb Plus) was added as a target molecular weight for the results at 1500 bp. The 16S rRNA gene sequencing procedure was then carried out at Laboratorium Penelitian dan Pengujian Terpadu (LPPT) in Yogyakarta using the PCR products. The isolate's forward and reverse sequences were then aligned using BioEdit software, and the resulting sequences were examined using the NCBI's BLAST program to examine their gene bank database.

2.2 Soymilk fermentation by *Weissella confusa* and *Lactiplantibacillus plantarum*

The chosen isolate and commercially purchased *Lactiplantibacillus plantarum* (Food and Nutrition Culture Collection, Universitas Gadjah Mada) were re-cultured in MRS broth for 24 hours at 37 °C. The isolate was then transferred into 30 mL of MRS broth, and a sample (1 mL) of the isolate and the commercially purchased *Lactiplantibacillus plantarum* were added, which was then placed into a shaker. The medium was maintained at 37 °C in a shaker incubator until its turbidity reached an optical density of 0.9 at 600 nm, indicating that, in all of the cultures utilized, there were around 10^7 CFU/mL of viable cell population. Centrifugation was used for 15 minutes at 5000 rpm to harvest the cultures. After discarding the media, the cells were resuspended and given two rounds of washing in sterile diluent to serve as the inoculum for soymilk fermentation. For a total of 6 hours, the inoculated soymilk was allowed to ferment; every two hours, the pH was measured using a universal indicator.

Using the plate count method on MRS agar the number of viable cells was determined. The plates underwent a 48-hour incubation period at 37 °C. To calculate the number of colony forming units (CFU)/mL of soymilk, the total plate count was calculated by counting the plates that contained between 25 and 250 colonies. This number was then multiplied by the dilution factor (Parseelan et al. 2019). The total lactic acid percentage was determined by the method as described by Gunawan et al. [6].

3 Results and discussion

3.1 Genomic identification of the isolated *Weissella confusa* from fermented soymilk by 16S rRNA

Figure 1 shows the single band at 1500 bp observed in the gel electrophoresis of the PCR product, which was produced by amplifying the isolate's DNA using universal 16S rRNA forward and reverse primers and a 1 Kb Plus marker. Since the primer has successfully amplified the DNA, a single band indicates that the test is considered successful. After that, the PCR product was transmitted to LPPT so that sequencing could be done. Following sequencing, the results were aligned to produce a consensus sequence. This sequence was then examined using NCBI's BLAST program, which compared it to species sequences stored in the GenBank database.

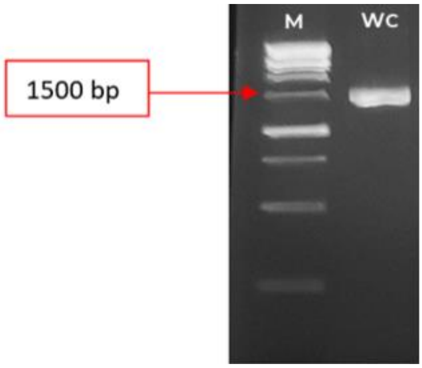

Fig. 1. PCR product electrophoresis after being amplified using 16S rRNA universal primers and 1 Kb plus marker; (M) marker, (WC) *Weissella confusa* isolate

From the BLAST results, it was possible to identify *Weisella confusa* strain NWAFU 8001 as the isolate, with a percent identity of 99.71% and a query cover of 100%. According to Spiegelhauer et al. [7] and Fessard & Remize [8], *Weisella confusa* is classified as a facultative anaerobic bacterium that is gram positive, has a coccoid colony shape, is non-motile, catalase negative, and does not form spores.

Physiologically, *Weissella confusa* is classified as a facultative anaerobic organism, meaning that because they can perform metabolism with or without oxygen, they can live in either aerobic or anaerobic environment. Macroscopically, colonies of *Weissella confusa* are observed as rods in pairs or chains [8]. Lactic acid, carbon dioxide, ethanol, and acetic acid are the end products of the fermentation process, which takes place when its metabolism proceeds in the absence of oxygen. *Weissella* confusa is known to grow in temperatures ranging from 15 °C to 37 °C, with certain strains being able to grow in temperatures as high as 47 °C.

The presence of *Weissella confusa* in a wide range of foods suggests that the species is adaptable to varying growth conditions in diverse environments. *Weissella confusa* has reportedly been isolated from a variety of sources, including fermented fruits, vegetables, dairy, and meat products [8].

3.2 Soymilk fermentation by *Weissella confusa* and *Lactiplantibacillus plantarum*

To compare the fermentation ability and determine the potential use of the isolated *Weissella confusa* isolate as a probiotic, in relation to a commercially available lactic acid bacteria, in this case *Lactiplantibacillus plantarum*, soymilk fermentation was carried out by inoculating a comparable quantity of viable cells from the *Weissella confusa* isolate and *Lactiplantibacillus plantarum* purchased commercially. Serial dilutions were performed to obtain the colony forming units (CFU)/mL following a fermentation for 6 hours at a temperature of 37 °C.

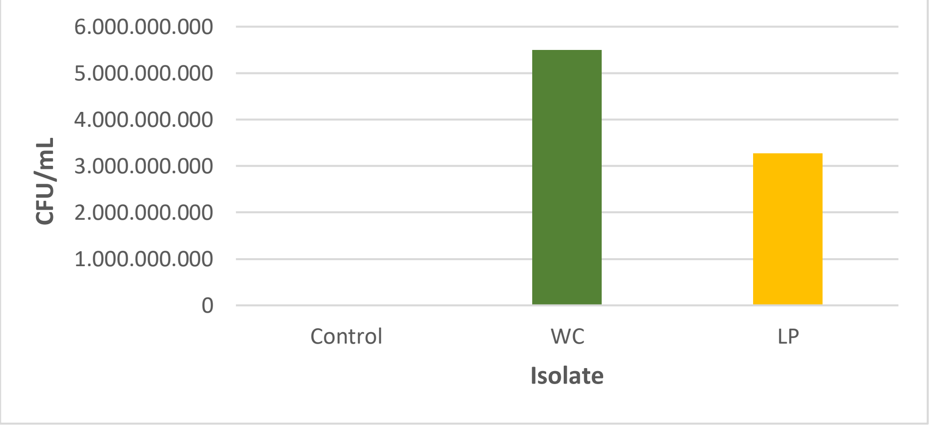

Fig. 2. Colony forming units (CFU)/mL of soymilk inoculated with 5% of *Weissella confusa* (WC) and *Lactiplantibacillus plantarum* (LP) for 6 hours at 37 °C

With a total of 5.5×10^9 CFU/mL, WC produced the greatest level of CFU/mL. On the other hand, the commercially purchased LP produced 3.27×10^9 CFU/mL. WC has a higher CFU/mL because it was isolated from soymilk, suggesting that it is well adapted to the growth conditions in soymilk. In contrast, commercially purchased LP may not have been isolated from a soymilk source, indicating that it is not fully adapted to the growth conditions. However, Chun et al.'s [9] similar study, which also employed *Lactiplantibacillus plantarum* and *Weissella confusa* for the 9–12-hour soymilk fermentation process, produced different outcomes, with *Lactiplantibacillus plantarum* achieving a higher CFU/mL than *Weissella confusa*. The reason for this discrepancy might be attributed to the varying strains of bacteria utilized. In this particular study, *Weissella confusa* was isolated from soymilk, which meant that it was well-suited to the growing conditions. The final CFU/mL of WC, which was used to ferment soymilk with the isolated bacteria, exceeded 10^6 CFU/mL, which is thought to be the minimum amount of bacteria needed in probiotic products to have a beneficial effect on the host [5].

Even though the use of bacteria to be considered as a probiotic bacterium has multiple criteria, such as the identification of the bacteria, its safety, the performance of the use of the bacteria in humans, and the viability of the bacteria throughout the use of the product, as described by Binda et al. [10], this study is limited to only analyzing the ability of the WC isolate to create a fermented product, through its viability in the fermented soymilk, its pH by the end of the fermentation process and its lactic acid percentage. Furthermore, in vivo studies of the use of WC as probiotics is still very limited, except for a study reported by Cupi and Elvig-Jørgensen [11], which analyzed the use of WC through oral administration in doses higher than human dosage for 90 days. The results showed that there were no proof of abnormalities when analyzed physically, behaviorally, or through blood tests. On the other

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hand, in vitro studies regarding the use of WC as probiotics has already been done in numerous studies, in which all reports showed that WC has the potential to be used as a probiotic bacterium through its tolerance to low pH, gastric juice, and bile, and its ability to adhere to intestinal epithelial cells [12,13]. Although in vitro results may support for the use of WC as probiotics for human consumption, further studies are required to assess the safety of its use.

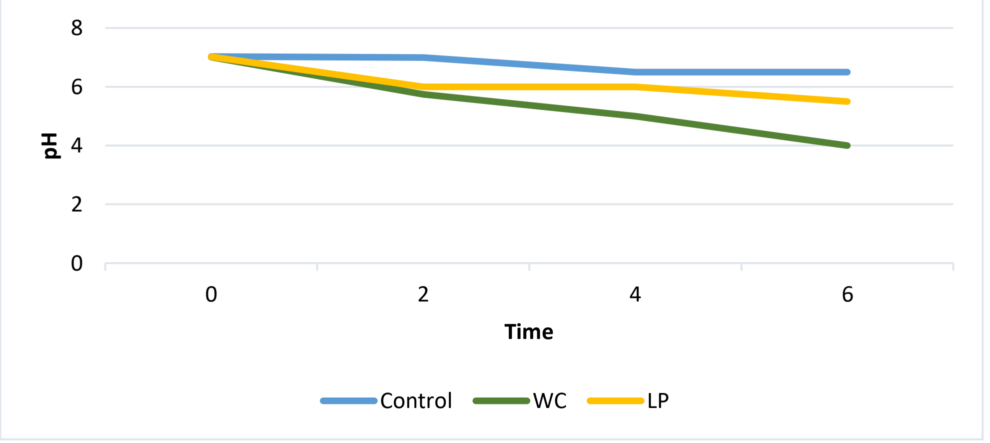

Fig. 3. Fermented soymilk pH inoculated with 5% of *Weissella confusa* (WC) and *Lactiplantibacillus plantarum* (LP) with a duration of 6 hours at 37 °C

A universal indicator was used to measure the soymilk's pH every two hours during the fermentation process in order to track the milk's changes in pH. A reduction in pH, as shown by Figure 3, often indicates the production of lactic acid due to the metabolism of lactic acid bacteria found in soymilk, indicating an ongoing fermentation process. From Figure 3, it can be inferred that WC caused the greatest pH decline, significantly lowering the milk's pH from the 2nd hour of fermentation in comparison to LP and the control. The reason for the variation in pH drop between WC and LP could be attributed to the pace at which the two isolates ferment the soymilk. WC may be better suited to the growth conditions and thus be able to ferment the soymilk more quickly than the commercially available LP, resulting in a higher production of lactic acid and a lower pH of the soymilk.

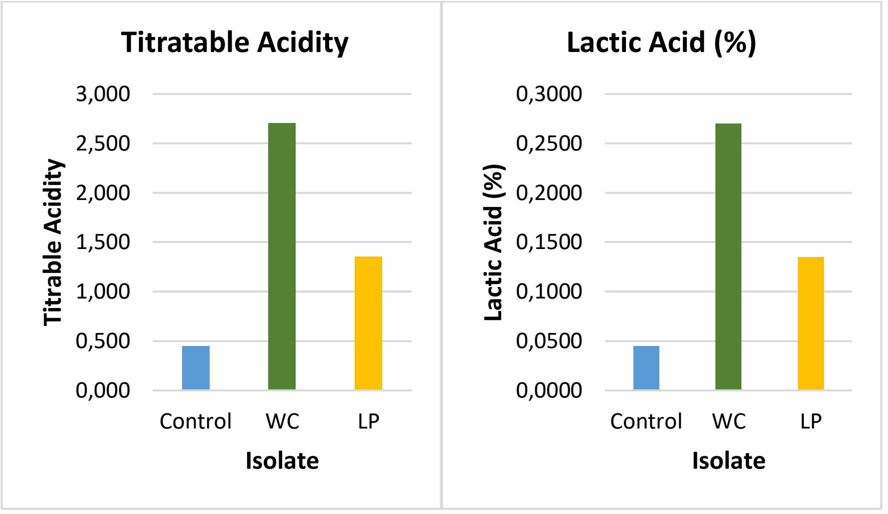

Fig. 4. Titratable acidity and total lactic acid content of the soymilk fermented with *Weissella confusa* (WC) *and Lactiplantibacillus plantarum* (LP)

By titrating a sample with two drops of phenolphthalein and continuing until a pink color is reached, the titratable acidity of a product may be measured using 0.1 N NaOH. According to Gunawan et al. [6] the total lactic acid content of the fermented soymilk is determined by measuring its titratable acidity. The overall amount of acid concentration in a product is essentially determined by the titratable acidity [14]. When compared with the titratable acidity, WC had the greatest titratable acidity, followed by LP, and then the control. This outcome is in line with the final pH of the fermented soymilk, which was reached in the same order as the lowest pH.

Furthermore, the total lactic acid percentage was determined using the formula reported by Gunawan et al. [6]. WC had the highest lactic acid percentage, followed by LP that was purchased commercially, and then the control. The variation in the overall lactic acid percentage value can serve as a predictor of the flavor produced by the various isolates throughout the fermentation process of soymilk. The flavor of the fermented soymilk could differ between one another as WC is heterofermentative while LP is homofermentative, in which WC has the potential to produce acetic acid through its fermentation process although organoleptic tests were not performed during this research [15]. Furthermore, because the pH of soymilk is lower with larger concentrations of lactic acid, fewer spoilage bacteria can develop in the product and therefore it is more difficult for it to deteriorate.

4 Conclusion

The *Weissella confusa* isolate obtained an overall better fermentation ability than the commercially bought *Lactiplantibacillus plantarum*, with higher number of CFU/mL, reaching 5.5×10^9 CFU/mL, a lower pH of 4, and a higher lactic acid content of 0.27%. Furthermore, both of the bacteria may have the ability to create a probiotic fermented soymilk product, as the number of viable bacteria in the product has reached above 10^6 CFU/mL. More research is necessary to determine the safety of using WC as probiotics for human consumption, even if in vitro data may support its usage.

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