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# Weissella paramesenteroides: A Lactic Acid Bacteria Producing Glutathione from Fermented Vegetable Based Keciwis Leaf

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**Abstract.** Glutathione (GSH), is one of the essential antioxidants to prevent free radicals and support the immune system. The low level of glutathione is associated with some diseases such as cancer, Alzheimer's, Parkinson's, and AIDS. Lactic acid bacteria (LAB) can produce glutathione. Indonesia, as a mega biodiversity country, has various vegetables and LAB sources that remain underexplored. This research aims to explore LAB-producing glutathione from fermented Panyileukan Bandung, 406142, West products derived from Indonesia's typical plant, the keciwis leaf. The method of this research includes sample preparation, lactic acid bacteria isolation, secondary metabolite production with cysteine precursor addition, glutathione analysis by Ellman's assay, identification of 16S rRNA gene of lactic acid bacteria, and glutathione qualitative analysis by HPLC. Fermented keciwis leaf-based products contain lactic acid bacteria with positive gram characteristics, can produce 0.33 - 0.37 mM extracellular glutathione. Precursor cysteine addition significantly increases glutathione (p<0,05). The selective lactic acid bacteria producing glutathione was identified as belonging to the genus Weissella, with a similarity value of 98.50%, closely related to Weissella paramesenteroides. Extracellular glutathione produced by this culture showed qualitative results on HPLC with a retention time of 6.34 minutes, indicating the presence of the sulfihydryl group. This study identified a new Indonesia source of lactic acid bacteria of Indonesian-origin lactic acid bacteria, specifically Weissella paramesenteroides, as a promising source for glutathione production.

Keywords: glutathione, keciwis, lactic acid bacteria, weissella

## Citation

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## INTRODUCTION

Keciwis leaves are abundant but underutilized vegetable commodities in Indonesia. Fermented vegetables are a source of lactic acid bacteria with potential health benefits. including the production of antioxidants such as glutathione. Glutathione is widely used in pharmaceutical, cosmetic and food industries (Schmacht et al., 2017). It is a tripeptide composed of three amino acids, y-glutamylcysteine, found in plants, animals, and microorganisms (Meister & Anderson, 1983). Glutathione plays diverse roles in biological systems for its antioxidative, immune boosting, and cellular detoxification activities (Wang et al., 2016). It helps maintain intracellular redox homeostasis, protecting the cells against oxidative damage. Low glutathione levels are associated with several diseases, such as cancer, Alzheimer's, Parkinson's, and AIDS (Jones, 2002).

Glutathione is widely found in eukaryotes and is the primary indigenous antioxidant in higher animals (Copley & Dhillon, 2002). Glutathione, a low molecular weight thiol, is produced by some lactic acid bacteria and other closely related gram-positive bacteria. Thiols are an important class of molecules that contribute to stress management bacteria in cells when they encounter various stress conditions in industrial processing or the gastrointestinal environment (Pophaly et.al., 2012). In prokaryotes, it is much distributed in gram-negative bacteria and only a few in Gram-positive bacteria. Lactic acid bacteria (LAB) are an essential group of microorganisms that have been domesticated to produce various fermented products, including fermented milk, cheese, sourdough, sausages, and fermented vegetables (Mozzi, 2015). Lactic acid bacteria are widely found in traditional Korean fermented food such as kimchi and in the human intestine (Ji et al., 2015). These bacteria represent the most dominant group among probiotic organisms, known for their specific health benefits to humans. More than 30 species of bacteria, including members of several different genera of lactic acid bacteria, have been identified in fermented vegetable kimchi. The primary microorganisms in kimchi fermentation are *Leuconostoc* (*Lc.*) *mesenteroides* and *Lactobacillus plantarum* (Kim et al., 2008).

Lactic acid bacteria have a high potential for glutathione biosynthesis and can effectively deliver glutathione molecules into the human system through a milk or wheybased functional fermented food (Pophaly et al., 2012). The Weissella genus has attracted significant research attention in recent years due to its probiotic and therapeutic potential, as well as its numerous applications in industries ranging from healthcare to skincare and food (Yadav et al., (2022), Teixeira et al., (2021). Indonesia has immense biodiversity as a source of lactic acid bacteria from plantbased fermented food. Approximately 194 lactic acid bacteria have been isolated from 21 types of fermented food Indonesia (Endang, 2018). Kiciwis leaf has not yet been analyzed as sourced from fermented vegetables. This research explores lactic acid bacteria from Indonesian plant-based fermented food, particularly from fermented keciwis leaf, and their potential for glutathione production.

## **MATERIALS AND METHODS**

## **Sample Preparation**

Samples were obtained from Indonesian plant-based fermented product manufacturers. Kimcis (keciwis leaf kimchi) was a Biotechnology Study Program Business product at the Universitas Muhammadiyah Bandung. Keciwis leaf kimchi is prepared from keciwis leaf vegetables, supplemented



with carrots, radishes, spring onions, and spices, fermented for three days. Samples were collected aseptically into falcone tube, homogenized and centrifuged.

## **Isolation of Lactic Acid Bacteria**

Lactic acid bacteria were isolated using MRS agar media supplemented with 1% CaCO<sub>3</sub>. Liquid samples of fermented keciwis leaves were serially diluted with physiological NaCl and then inoculated using the pour method, followed by the four-way streak technique. The cultures were incubated at 37°C for 24 hours. The isolation results were based on obtaining a pure isolate from the four-way technique and observing the morphology of isolates, including the presence of clear zones on MRS agar and 0.5% CaCO<sub>3</sub> (Meidong et al., 2017).

## **Production of Secondary Metabolites**

Starter cultures from each isolated colony were sampled and inoculated into MRS broth in 100 mL Erlenmeyer flasks with a medium volume of 25 mL. The cultures were incubated in a shaking incubator at 150 rpm and 37°C for 24 hours. A 1% culture starter was inoculated into MRS liquid medium and grown in production media supplemented with 1.5% glucose and 1% mineral MgSO<sub>4</sub>. Cultures were incubated at 200 rpm 30°C for 48 hours. Cysteine precursor was added at 12 hours with a concentration of 0.025 M. The secondary metabolites were obtained by centrifugation at 10.000 x g, 4°C for 10 minutes (Fernándes & Steele, 1993). The supernatant was separated to analyze the glutathione content.

## **Glutathione Analysis**

Glutathione content was tested using Ellman's method with 5,5'-dithiobis(2-nitrobenzoic acid) or DNTB reagent. The

reaction buffer was 0.1 M phosphate buffer at pH 8. The test procedure and preparation of standard solutions are described by (Ellman (1959) and Cribb et al. (1989). The reaction buffer solution was sodium phosphate 0.1 M as much as 4 mg/mL. Ellman's reagent was dissolved in 1 mL buffer solution. Each 250  $\mu$ l was placed into a 96-well plate, then 50  $\mu$ L Ellman's reagent was added, homogenized, incubated at room temperature chamber for 15 minutes, and then measured at a 412-500 nm wavelength. The standard solution uses cysteine hydrochloride monohydrate MW-175.6 in various concentrations by making a standard curve.

## **Glutathione Qualitative Analysis**

HPLC analysis was performed following the method described by Appala et al., (2016). Glutathione derivatization was conducted using Ellman's reagent. The sample was aliquoted to 0.5 mL portion, and then 0.5 mM Ellman's reagent was added. The mixture was incubated for 20 minutes at 60°C, followed by the injecting a 10 ul sample at a flow rate of 0.8 mL/min. The analysis had a runtime of 20 minutes, with detection at a wavelength of 280 nm. The HPLC machine was a water e2695 separation module with a 2489 UV/Vis detector. The mobile phase used ten mM phosphate buffer pH 2.5. The column used is C-18, with a length of 250 mm and a diameter of 4.6 mm.

# Molecular Identification of Lactic Acid Bacteria by 16S rRNA PCR

DNA from the selected bacterial strain with the highest glutathione content was isolated using Bio-Rad<sup>TM</sup> DNA isolation kit, and the presence of DNA was confirmed by agarose gel electrophoresis. The bacterial DNA was identified by 16S rRNA gene amplification using PCR with 25 cycles.

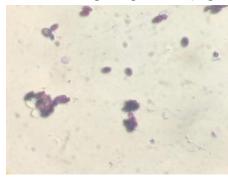
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The PCR protocol included pre-denaturation at 95°C for 2 minutes, denaturation at 95°C for 1 minute, annealing at 50°C for 1 minute, elongation at 72°C for 1 minute, and final elongation at 72°C for 10 minutes—characterization of PCR results by agarose gel electrophoresis. DNA sequences were analyzed using the Sanger method on the Biosystem 3130 and 3130xl genetic analyzer. Sequencing results were processed and interpreted using a bioinformatics approach with bioedit software (Akihary & Kolondam, 2020).

#### RESULTS AND DISCUSSION

Eight lactic acid bacteria colonies were isolated from fermented vegetable samples based on keciwis leaves. The isolates form clear zones on MRS agar media supplemented with CaCO<sub>3</sub>, indicating acid production that reacts with base substances. Growing bacterial isolates were identified based on colony morphology, including shape, color, margins, and elevation (Sousa et al., 2013). The colonies were predominantly round, white, and convex in elevation, with an entire margin. Simple microbiological tests, including gram staining, confirmed all eight gram-positive isolates. The cells of the family Lactobacillaceae are gram-positive, non-spore-forming facultative or strict anaerobic bacteria. Coccoid or rod-shaped cells may form chains, pairs, or tetrads (genus Pediococcus) (Zheng et al., 2020). Weissella genus was divided into 16 species with many similar phenotypes, the delimitation of which is based primarily on genetic analysis. Weissella hellenica, Weissella paramesenteroides, and Weissella thailendensis are cocci; the other species are small bacilli or coccoid bacilli (Lonvaud-Funel, 2014). Based on gram staining and morphological observations, the isolated lactic acid bacteria exhibited bacillus characteristics and were confirmed as gram-positive (Figure 1).



**Figure 1.** Gram staining lactic acid bacteria from kimchi keciwis with 1000x magnification

Metabolite production from the LAB isolate was performed using 1% culture starter in production medium. The medium consisted of MRS broth enriched with 1.5% glucose as a carbon source and 1% MgSO<sub>4</sub> as a mineral supplement. Various treatments were applied, including adding the precursor amino acid cysteine (0.025 M) at 12 hours. After 24 hours, extracellular glutathione was harvested by centrifugation at 10,000 x g, 4 oC, for 10 minutes (Fernándes & Steele, 1993).

Glutathione concentration was determined using Ellman's method with a DNTB reagent (5,5-dithio-bis-(2-nitrobenzoic acid)), in a 0.1 M phosphate buffer at pH 8. The reaction between DNTB and the cysteine group in glutathione produces an orange-colored complex, measured spectrophotometrically at an absorption wavelength of 412–500 nm. The results of Ellman's assay for the eight LAB isolates Ellman's assay are presented in table 1.

A T-test revealed a significant difference in glutathione concentration between LAB isolated from keciwis leaf kimchi with and without the addition of cysteine precursor (p<0.05). Adding the cysteine precursor resulted in a higher glutathione concentration than the sample without the precursor (Table 1).



**Table 1.** Concentration of glutathione (mM) of lactic acid bacteria without and with the addition of cysteine precursors

Lactic Acid Bacteria Isolates	mM Glutathione (with cysteine precursors)	Mean (mM)	mM Glutathione (without cysteine precursors)	Mean (mM)
LAB-1	0.3580		0.3615	
LAB-2	0.3583		0.3514	0.343
LAB-3	0.3385	0.358	0.3776	
LAB-4	0.3383	0.550	0.3646	
LAB-5	0.3402		0.3461	
LAB-6	0.3348		0.3545	
LAB-7	0.3366		0.3494	
LAB-8	0.3385		0.3619	

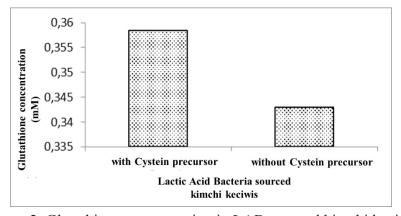
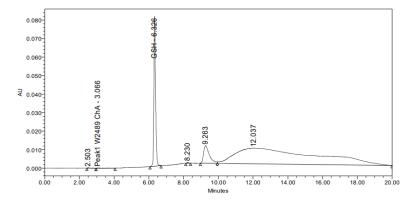


Figure 2. Glutathione concentration in LAB-sourced kimchi keciwis



	Peak Name	RT	Area	% Area	Height
1		2.503	4131	0.12	279
2	Peak1 W2489 ChA	3.066	5826	0.16	249
3	GSH	6.326	548195	15.41	80786
4		8.230	3340	0.09	450
5		9.263	217505	6.11	9451
6		12.037	2779481	78.11	8452

Figure 3. Cysteine Standard HPLC chromatography 100 ppm



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The efficiency of glutathione production relies on the biotransformation effectiveness of amino acid precursors (Schmacht et al., 2017). Researchers have extensively studied the addition of amino acid precursors, with cysteine, glutamate, and glycine being the most commonly used. Among these, cysteine is the rate-limiting precursor (Schmacht et al., 2017), (Wang et al., 2016). The addition of cysteine significantly increases glutathione concentration compared to controls without precursors. As the rate-limiting amino acid in the de novo synthesis of glutathione (GSH), L-cysteine supplementation represents a potential strategy to enhance GSH levels and mitigate oxidative stress associated with various diseases (Gould & Pazdro, 2019).

Previous studies have analyzed glutathione content in lactic acid bacteria. Yoon & Byun (2004) reported that the intracellular free extract of *Lactobacillus* spp. cells contain significant levels of glutathione. All Lactobacilli strains analyzed had at least 7.4 µmol/g of glutathione sulphydryl in their cytoplasm. Seventeen strains were categorized into three statistically distinct groups based on their intracellular glutathione levels (p>0.01). Among these, *L. casei* HY 2782, L. rhamnosus CU 01, and L. plantarum CU 03 exhibited the highest glutathione concentrations, exceeding 14.0 µmol/g glutathione sulphydryl. Notably, L. casei HY2782 demonstrated the highest glutathione content at 25.1 umol/g. Moderate levels of cellular GSH were detected in the cytoplasm of L. casei CU 026, L. acidophilus 4356 and L. brevis CU 05, with an average content of 12.0 µmol/g glutathione sulphydryl. Another strain group was classified as low GSH, containing less than 12.0 µmol/g. Fernándes & Steele (1993) also reported thiol group concentrations ranging from 0.7 to 81 nmol/mg of protein and glutathione concentrations between 6 to 51 nmol/ mg of protein. The highest concentrations

were found in strains of *Lactococcus lactis* ssp. cremoris and *Streptococcus thermophilus*, followed by *Leuconostoc mesenteroides* ssp. cremoris. Based on these findings, extracellular glutathione produced by lactic acid bacteria isolates is relatively high. Extracellular glutathione was optimized by Rollini et al. (2010) in *Saccharomyces cerevisiae*. Further analysis is required to evaluate the intracellular glutathione levels in these isolates.

Glutathione derivatization was performed using Ellman's reagents. A 0.5 mL aliquot of the sample was mixed with 0.5 mM of Ellman's reagent and incubated for 20 minutes at 60°C. Subsequently, 10 µl of the sample was injected at a flow rate of 0.8 mL/min, with a total runtime of 20 minutes, and detection was performed at a wavelength of 280 nm. The HPLC system was the Waters e2695 Separation Module with 2489 UV/Vis detector. The motion phase consisted of a 10 mM phosphate buffer at pH 2.5. The column employed was a C-18 type, measuring 250 mm long and 4.6 mm in diameter.

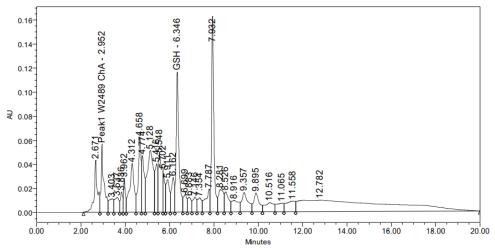
The sulfhydryl group resulting from the derivatization of the standard solution was observed at a retention time of 6.325 minutes (Figure 3). The HPLC analysis of the sample showed that the glutathione content had a nearly identical retention time of 6.346 minutes (Figure 4). These findings align with the glutathione analysis reported by (Appala et al., 2016), which identified a retention time of 11.23. This difference is attributed to the variation in the HPLC column used. This study employed a C-18 column, while the reference utilized a C-8 column. Different columns influence the retention of compounds within the matrix, resulting in various retention times. The HPLC analysis of the samples indicated that the Glutathione content exhibited a similar retention time of 6.346 minutes. While this HPLC test provides a reliable basis for qualitative analysis, further confir-



mation can be achieved using LC-MS/MS.

Amplicon genes of 16S rRNA obtained from PCR were sequenced to determine the sequence of nucleotide bases. DNA sequencing involves determining a DNA molecule's exact sequence of nucleotide bases. Sequencing of the 16S rRNA gene using forward primers obtained 1,240 bases, while reverse primers produced 871 nucleotide bases. After obtaining the consensus sequence, a BLAST analysis was performed using NCBI. The BLAST results revealed that the LAB isolate Keciwis-8 sample had 98.50% identity to Weissella paramesenteroides.

Phylogenetic tree construction was performed using 16S rRNA sequence of the samples with MEGA11 software. The purpose of constructing the phylogenetic tree was to analyze the evolutionary relationship between the sample organisms and reference organisms based on their sequences obtained from the NCBI website (www.ncbi.nlm.nih. gov). The phylogenetic trees were built using comparable sequences retrieved from the BLAST results on the NCBI site. The comparison sequences are listed in Table 2.



**Figure 4.** HPLC Chromatography Sample BAL-8 (Weissella paramesenteroides)

**Table 2.** Taxon of comparative organisms with the axial number of the NCBI

Taxon	Accession number		
Weissella paramesenteroides strain 120	FJ654452.1		
Weissella hellenica strain SJKO10-2	MN176361		
Leuconostoc paramesenteroides strain 1204	FJ654458.1		
Enterococcus saccharolyticus subs. saccharolyticus strain NCDO 2594	NR11929.1		



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Weissella spp is a non-spore-forming, heterofermentative facultative bacteria that are Gram-positive, catalase-negative, and short-rod-shaped (Kamboj et al., 2015). They are classified as lactic acid bacteria (LAB) in the phylum Firmicutes, class Bacilli, order Lactobacillales, and family Leuconostocaceae (Fusco et al., 2015). These bacteria are usually isolated from fermented foods such as sausages, pickled Korean Kimchi, and barrels used to produce Japanese pickles. While some strains have been observed as probiotics, others have not (Teixeira et al., 2021). Keciwis is a member of the cabbage group (Brassicaceae). Lactic acid bacteria isolated from red cabbage fermentation have shown 100% similarity to Weissella cibaria and Weissella confusa (Rambitan et al., 2018). Additionally, Weissella cibaria has been identified in red pepper powder during Kimchi fermentation (Kang et al., 2016).

The Weissella strain has been extensively researched due to its technological potential and probiotics potential. Certain strains have garnered attention from the pharmaceutical, medical, and food industries for their ability to produce antimicrobial exopolysaccharides (EPS). In addition, Weissella strains help protect food from pathogens by producing bacteriocins, hydrogen peroxide, and organic acids. The genus Weissella has also demonstrated potential in treating atopic dermatitis and certain cancers. Notable strains, such as W. cibaria, W. confusa, and W. Paramesenteroides, have been studied for their probiotic capabilities, including their fermentation of prebiotic fibers and their ability to survive in the gastrointestinal tract (Fusco et al., 2015). However, the genus Weissella as a producer of Glutathione has yet to be discovered, making the finding of this study highly novel.

## **CONCLUSION**

The study aimed to isolate and identify lactic acid bacteria from Keciwis leaf-based fermented vegetables capable of producing glutathione. This study obtained eight lactic acid bacteria isolates and produced extracellular glutathione concentrations ranging from 0.33 - 0.37 mM. Adding a cysteine precursor significantly increased glutathione concentration compared to culture samples without the precursors (p<0.05). Molecular identification revealed 98.5% similarities with Weissella paramesenteroides. with a bootstrap value of 75%. Qualitative testing using HPLC revealed that extracellular glutathione produced by keciwis-8 lactic acid bacteria sample appeared at a retention time of 6.34 minutes, identified as sulfhydryl group. This study identified a novel source of lactic acid bacteria from Indonesian vegetables, highlighting its potential for glutathione production. This study can inspire further exploration to enhance Indonesian microbial biodiversity, particularly lactic acid bacteria, for developing probiotic candidates applicable in health and the food industry.

## **AUTHOR CONTRIBUTION**

L.H.M contributed to the microbiology and biochemistry field (lactic acid bacteria isolation and glutathione assay), study literature and corresponding author. H assisted in the statistical data analysis. W.P assisted in molecular identification and article translating. N.H. assisted in analyzing bioinformatics and HPLC results. I.M. assisted in microbial analysis and literature management.



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## **CONFLICT OF INTEREST**

The authors declare that there is no conflict of interest regarding this paper's publication.

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