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Research Article

Comparison of Characteristics and Antibacterial Activity of Mandai Cempedak Vinegar with Variations in Fermentation Methods

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Abstract

Mandai, a traditional fermented food product from East Kalimantan, is derived from the edible mesocarp of the cempedak (*Artocarpus champeden*) fruit. This ancestral fermentation technique, passed down through generations, is known to produce lactic acid bacteria (LAB) with potential probiotic properties. This study aimed to characterize LAB isolated from mandai vinegar and evaluate their antibacterial activity under two distinct fermentation conditions: a defined starter culture of *Lactobacillus casei* strain Shirota and spontaneous fermentation in a 10% NaCl solution. The latter, referred to as salt fermentation, yielded three bacterial colonies, while the starter culture method produced four. All isolates from both methods shared similar morphological characteristics, including a rod shape, white coloration, entire margins, and convex surfaces. Further analysis confirmed they were Gram-positive, catalase-negative, and indole-negative, with the ability to ferment various carbohydrates. While all LAB isolates demonstrated generally low antibacterial activity against *Escherichia coli* ATCC 25922, isolate CML 1 exhibited the highest antibacterial potential. These findings highlight the potential of mandai vinegar as a source of functional LAB, warranting further investigation into its applications as a functional food ingredient.

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INTRODUCTION

Fermentation with lactic acid bacteria (LAB) is fundamental to Indonesian traditional culture, yielding a wide range of fermented foods. Among these is mandai cempedak (*Artocarpus champeden*), a fermented delicacy from East Kalimantan made by brining the inner skin of the cempedak fruit. This method not only preserves the food and repurposes a fruit byproduct but also encourages the growth of probiotic-producing LAB[1-3]. The fermentation is characterized by a dynamic interplay of LAB population shifts, sugar reduction, and the production of organic acids, all of which are key indicators of the process's progression[4,5]. The probiotic potential of mandai is a significant health benefit, with several isolates, particularly *Lactobacillus plantarum*, showing potent antibacterial activity against pathogens like *Listeria monocytogenes*, *Escherichia coli*, and *Salmonella typhimurium*[1-3].

A major drawback of traditional mandai fermentation is the high salt content (15–25%), which poses health risks from excessive sodium intake. A viable alternative is the use of a starter culture, such as *Lactobacillus casei* strain Shirota, which accelerates the fermentation and allows for a rapid increase in the LAB population, reaching 99% of the microbial community by day seven at a pH of 3.5[2]. *Lactobacillus casei* strain Shirota is well-suited for this purpose due to its ability to survive harsh gastrointestinal conditions and its capacity to enhance immune responses[6]. It also produces antimicrobial

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compounds effective against pathogens like *Salmonella* sp. and *Staphylococcus aureus*[7], and has been shown to reduce cholesterol levels in Indonesian studies[8], making it an ideal candidate for enhancing the functional properties of mandai.
While the probiotic potential of mandai from *A. champeden* is recognized, that of its derivative, mandai vinegar, has not been extensively studied. Mandai vinegar is produced from fermented *A. champeden* peel, with its characteristic properties derived from volatile organic acids like lactic, propionic, and butyric acids[2,9]. LAB, which are "generally recognized as safe" (GRAS), produce antimicrobial substances such as hydrogen peroxide and bacteriocins that inhibit food-spoiling microorganisms[10]. Leveraging this potential, the creation of mandai vinegar offers a novel and natural source of probiotics. This study aims to compare the properties of LAB isolates from mandai vinegar fermented with a starter culture to those from traditionally fermented mandai vinegar. We will also test the antibacterial activity of isolates from both fermentation methods against *E. coli* ATCC 25922.

MATERIALS AND METHODS

Materials

The materials utilized in this study included *A. champeden* fruit peel, distilled water, NaCl 0.9%, $CaCO_3$, H_2O_2, crystal violet, I_2, 96% ethanol, and safranin. All chemicals and reagents were of analytical grade. Microbial media used were deMan Rogosa Shaepe Agar (Merck) and deMan Rogosa Shaepe Broth (Merck). Bacterial strains included a commercial *L. casei* strain Shirota starter culture and a standardized *E. coli* ATCC 25922 strain. A range of laboratory equipment was used for the experiments. These included an autoclave (GEA LS-50HD) for sterilization, a Laminar Air Flow (Biobase) cabinet for aseptic procedures, an incubator for bacterial growth, and an analytical balance (Mettler Toledo) for precise measurements. General laboratory glassware and tools such as glass jars, Petri dishes (Pyrex®), Erlenmeyer flasks (Pyrex®) 250 mL, beakers (500 mL and 100 mL), measuring cylinders (Iwaki®) 100 mL and 50 mL, dropper pipettes, a cotton spatula, and an inoculating loop were also employed. Microscopic analysis was performed using an Olympus CX23® microscope, microscope slides, and cover glass. A Bunsen burner and matches were used for flame sterilization.

Methods

Fermentation of mandai vinegar with starter culture

Artocarpus champeden peel used for mandai vinegar production was taxonomically identified at the Laboratory of Plant Anatomy and Systematics, Universitas Mulawarman (Certificate No. 168/UN17.8.5.1/FMIPA/LL/VII/2025). Following identification, the peel was thoroughly cleaned and chopped into small pieces. To extract the sap, the chopped peel was boiled at 100°C for five minutes, and this process was repeated twice to ensure complete sap removal. The peel was then drained and allowed to cool to room temperature (28±3°C).
For fermentation, 100 g of the prepared peel was placed in a covered container and combined with boiled water at a 2:1 ratio. The mixture was inoculated with a 4% (v/v) *L. casei* starter culture and fermented for 14 days at a controlled low temperature of 8°C. After fermentation, the mixture was filtered through a sterile cloth to separate the solids. The resulting liquid was then centrifuged at 3000 rpm for 15 minutes at room temperature to obtain the supernatant, which is the final mandai *A. champeden* vinegar. The finished product was stored at 5-8°C to maintain its stability[9].

Fermentation of mandai vinegar with salt

The preparation of mandai *A. champeden* vinegar via salt-fermentation begins with the careful separation of the fruit's inner (mesocarp) and outer (exocarp) peels. A total of 500 g of the inner peel was thoroughly cleaned and sliced. To remove any residual sap and mucilage, the sliced peel was boiled in distilled water at 100°C for five minutes, a process that was repeated twice until the peel was free of these substances. After boiling, the peel was drained and allowed to cool to ambient temperature (28 ± 3°C) before being placed into a sterile jar. To initiate fermentation, the peel was submerged in 1.2 L of distilled water with the addition of a 25% (w/v) salt concentration. The mixture was then incubated at room temperature for 14 days to produce mandai. Following fermentation, the resulting mandai was crushed and filtered using a sterile cloth. The liquid was then centrifuged at 3000 rpm for 15 minutes at room temperature to obtain the clear supernatant, which constitutes the mandai vinegar. The final sample was stored in a refrigerator at 5–8°C to maintain its stability.

Isolation of LAB vinegar from A. champeden

The isolation of LAB from mandai vinegar was performed using the following procedure. First, 0.5 mL of the vinegar was aseptically transferred to 4.5 mL of 0.9% NaCl solution, followed by serial dilutions ranging from 10^{-1} to 10^{-6}. Subsequently, 1 mL from each dilution was poured into Petri dishes containing MRS agar supplemented with 0.5% $CaCO_3$. These plates were then incubated at 37°C for 48 hours. Colonies that exhibited a clear zone, indicating acid production, were selected and re-streaked onto fresh MRS agar plates to obtain pure, uniform colonies. These pure colonies were then inoculated into MRS broth and incubated under identical conditions. For long-term storage and use as stock cultures, the isolates were transferred to MRS broth tubes containing 0.2% agar and $CaCO_3$. All isolates were maintained on MRS slant agar for subsequent macroscopic and microscopic characterization[11]. For clarity and ease of reference, isolates obtained from the traditional salt-fermented vinegar were designated as CMG, while those from the vinegar fermented with the *L. casei* strain Shirota starter culture were designated as CML.

Characterization of LAB from mandai vinegar using Gram staining

Gram staining was performed to characterize the bacterial isolates. A smear of the isolate was prepared on a microscope slide and then stained with crystal violet for two minutes before being rinsed with sterile distilled water. To intensify the stain, Lugol's iodine was applied for two minutes, followed by another wash with distilled water. The smear was then decolorized using 96% alcohol before a counterstain with a 0.5% safranin solution was applied for one minute. After a final rinse and air-drying, the bacterial coloration was observed and characterized microscopically to determine its Gram status[11].

Characterization of LAB from mandai vinegar using catalase test

The catalase test was performed to determine the ability of LAB isolates to produce the catalase enzyme. For the characterization, one to two drops of 30% H_2O_2 were added to 24-hour-old LAB isolates on a microscope slide using an inoculation loop. The formation of bubbles upon the addition of H_2O_2 indicated a positive result, confirming the presence of catalase activity in the isolates[12].

Characterization of LAB from mandai vinegar using indole

The indole test was conducted to determine the ability of LAB to metabolize the amino acid tryptophan into indole derivatives. The assay was performed by adding 3–5 drops of Kovac's reagent to the surface of the bacterial culture medium. A positive result was indicated by the formation of a red ring on the medium's surface[13].

Characterization of LAB from mandai vinegar using carbohydrate fermentation test

Carbohydrate fermentation was assessed by inoculating LAB isolates into Nutrient Broth (NB) medium. This medium was specifically supplemented with various carbon sources—sucrose, glucose, and lactose—and the pH indicator phenol red. The inoculated cultures were then incubated at 37 °C for 24 hours. Phenol red serves as a visual indicator of acid production: the medium is initially red at a neutral pH (~7.0), transitions to orange as the pH slightly decreases, and becomes yellow under acidic conditions (pH <6.8). A positive fermentation result was indicated by a color change from red to orange or yellow, signifying that the LAB isolates successfully fermented the carbohydrate and produced acid. Conversely, a medium that remained red after incubation was considered a negative result, indicating no fermentation. An uninoculated control was included for each test to ensure the medium's initial color and pH stability[12].

Evaluation of antibacterial efficacy against E. coli ATCC 25922

The antibacterial activity of the LAB isolates was evaluated using the paper disk diffusion method. Prior to testing, the pathogenic bacterium *E. coli* ATCC 25922 was cultured in BPW broth for 18 hours at 37°C. The bacterial suspension's turbidity was adjusted to the 0.5 McFarland standard, equivalent to approximately 1.5×10^8 CFU/mL. A volume of 100 µL of the standardized *E. coli* suspension was uniformly spread across the surface of a Mueller-Hinton Agar (MHA) plate using a sterile glass rod. Subsequently, sterile paper disks (6 mm in diameter) were placed on the inoculated MHA plates after being impregnated with 25 µL of the respective LAB culture, which had also been grown in MRS broth for 18 hours. The plates were then incubated for 24 hours at 37°C. Following the incubation period, the average diameter of the inhibitory zones was measured in three different orientations. All tests were conducted in duplicate to ensure the reliability and reproducibility of the results[14].

Data analysis

Lactic acid bacteria isolate from mandai vinegar were characterized using a descriptive approach, which involved macroscopic, microscopic, and biochemical analyses. All data regarding the isolates' characteristics were compiled into a table. For identification, a profile-matching method was employed, comparing the observed traits with the key characteristics documented in Bergey's Manual® of Systematic Bacteriology (2nd Edition)[15] and Bergey's Manual® of Determinative Bacteriology (9th Edition)[16]. To investigate the antibacterial activity of the LAB isolates against the test bacteria, the diameters of the inhibition zones were measured. The collected data were organized and statistically analyzed using Statistical Product and Service Solutions (SPSS) software. A one-way ANOVA test was conducted to determine statistical significance, followed by a T-test for post-hoc analysis.

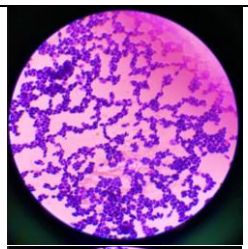
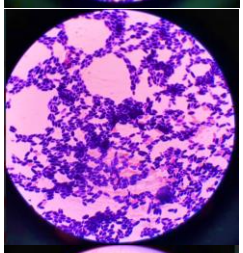
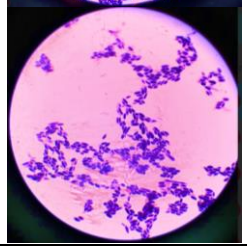
RESULTS AND DISCUSSION

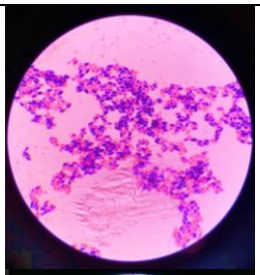
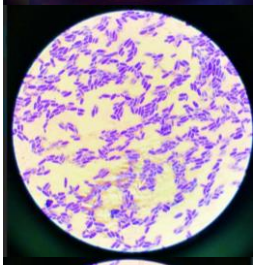
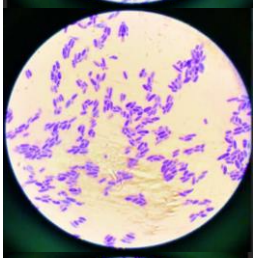
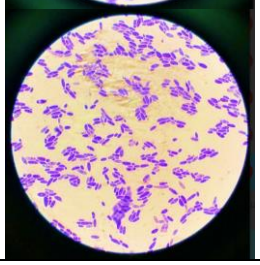
Fermentation is a biochemical process where microorganisms or their enzymes convert organic materials. This process can be categorized into two methods based on the microbial source: spontaneous fermentation and starter culture fermentation. Spontaneous fermentation relies on naturally occurring microbes to initiate the process, while starter culture fermentation involves the deliberate addition of specific microorganisms, such as LAB, which are widely used in fermenting various foods. The quality of the final product is heavily influenced by the activity of these LAB, and their growth patterns can be observed in both spontaneous and starter-based fermentations when the process is successful[17].

This study focused on mandai vinegar; a derivative of fermented *A. champeden*. The fermentation of *A. champeden* fruit peel is known to produce various organic acids through microbial activity, with lactic acid being a key contributor to flavor enhancement. Vinegar, a product of fermentation, is traditionally used to improve food flavor, preserve meat, and enhance palatability[9,18].

In our investigation, we successfully isolated and purified three LAB strains from spontaneously fermented mandai *A. champeden* vinegar and four strains from mandai vinegar fermented with a starter culture. The isolation and purification were confirmed by the distinct appearance of clear zones on MRS agar supplemented with 1% $CaCO_3$, indicating acid production by the LAB. The detailed characterization and representative images of these isolates are presented in **Table I**.

Table I. Characteristics and biochemical tests of *Lactobacillus* isolates from mandai vinegar.

Fermentation method	Isolate code	Microscopic	Biochemical characteristics	Morphological and Gram stain characteristics
Traditional salt-fermentation	CMG 1		Catalase-negative, indole-negative, and able to ferment sucrose, lactose, and glucose.	Cream-colored, circular, convex, and small colonies with entire edges; bacillus shape and Gram-positive.
	CMG 2		Catalase-negative, indole-negative, and able to ferment sucrose, lactose, and glucose.	Cream-colored, circular, convex, and small colonies with entire edges; bacillus shape and Gram-positive.
	CMG 3		Catalase-negative, indole-negative, and able to ferment sucrose, lactose, and glucose.	Cream-colored, circular, convex, and small colonies with entire edges; bacillus shape and Gram-positive.

Fermentation with *L. casei* strain Shirota	CML1		Catalase-negative, indole-negative, and able to ferment sucrose, lactose, and glucose.	Cream-colored, circular, convex, and small colonies with entire edges; bacillus shape and Gram-positive.
	CML2		Catalase-negative, indole-negative, and able to ferment sucrose, lactose, and glucose.	Cream-colored, circular, convex, and small colonies with entire edges; bacillus shape and Gram-positive.
	CML3		Catalase-negative, indole-negative, and able to ferment sucrose, lactose, and glucose.	Cream-colored, circular, convex, and small colonies with entire edges; bacillus shape and Gram-positive.
	CML4		Catalase-negative, indole-negative, and able to ferment sucrose, lactose, and glucose.	Cream-colored, circular, convex, and small colonies with entire edges; bacillus shape and Gram-positive.

The identification of *Lactobacillus* isolates from mandai vinegar was achieved through a profile-matching approach, which involved comparing their observed characteristics with key properties documented in Bergey's Manual® of Systematic Bacteriology[15]. All isolates were found to be Gram-positive, catalase-negative, and indole-negative, with a predominant bacillus (rod-shaped) morphology. Seven of the strains, characterized by creamy-white colonies, rod-shaped cells, and convex elevations, were classified within the genus *Bacillus*[19].

Microscopic examination of all isolates, stained with the Gram method at 100x magnification, revealed a purple color, confirming their Gram-positive status due to the retention of crystal violet within their thick peptidoglycan cell walls. Morphologically, the rod-shaped cells were observed in various arrangements, including pairs and small clusters. No endospores were detected. The ability of Gram-positive bacteria to retain the stain is attributed to their robust peptidoglycan structure, a three-dimensional network of amino sugars, which becomes dehydrated by the acetone-alcohol decolorizing agent, acting as a permeability barrier[20].

All isolates tested negative for the catalase biochemical test, as evidenced by the absence of bubbles after the addition of hydrogen peroxide. This indicates that these LAB isolates do not synthesize the catalase enzyme, which is consistent with their microaerophilic or obligate anaerobic nature. The lack of this enzyme means they cannot decompose toxic hydrogen peroxide, which consequently inhibits their growth in the presence of oxygen[21,22].

The subsequent biochemical tests for carbon fermentation were positive for all isolates, indicated by a color change from red to yellow, suggesting their ability to utilize various carbon sources. Conversely, all isolates tested negative for the indole test; no red ring formed after adding Kovac's reagent, confirming the absence of the tryptophanase enzyme. LAB typically lacks this enzyme, which prevents them from metabolizing tryptophan as an energy source[11].

The collective results of this analysis confirm that LAB isolated from both fermentation methods belong to the genus *Lactobacillus*. This finding is consistent with previous research, which identified *L. plantarum* in salt-fermented mandai and

confirmed the presence of *L. casei* when a starter culture was used[1]. As a genus, *Lactobacillus* is recognized as GRAS and is widely used in the food and health industries for its probiotic properties and adaptability to diverse environmental conditions[23].

Finally, all isolates were tested for antibacterial activity against *E. coli*. The presence of a clear zone around the paper disks demonstrated the isolates' antibacterial efficacy. The measurements of these clear zones are detailed in **Table II**, providing quantitative evidence of the isolates' antimicrobial potential.

Table II. Average diameter of LAB inhibition zone of mandai vinegar isolate against *E. coli*.

Samples	Inhibition zone (mm)
CMG 1	2.97 ± 0.22^{a}
CMG 2	2.89 ± 0.28^{ab}
CMG 3	2.67 ± 0.23^{abc}
CML 1	3.75 ± 0.44^{abde}
CML 2	3.28 ± 0.42^{acde}
CML 3	3.67 ± 0.15^{abcde}
CML 4	3.52 ± 0.41^{abcde}
Negative control	0.000

Note: Values are presented as the mean ± SD of three replicates. Data with superscripts (a–e) indicate significant differences ($p < 0.05$) based on one-way ANOVA followed by Tukey's post hoc test.

The analysis of the inhibition zones revealed that the LAB isolates from mandai vinegar exhibited a general ability to inhibit the growth of *E. coli*. Specifically, isolate CML 1 showed the most significant inhibition zone. However, based on the established criteria for antibacterial efficacy, where an inhibition zone less than 5 mm is classified as weak, the overall efficacy of the tested isolates from cempedak mandai vinegar is considered low[24]. The size of the inhibition zone is directly influenced by the efficacy of the probiotic bacteria and the quantity of bacterial cells that can inhibit pathogenic growth. A clear, distinct inhibition zone is often indicative of bacteriocin activity, a proteinaceous toxin that acts via a "single-hit inactivation" mechanism, where one molecule can inactivate one target cell[25].

The antibacterial potential of LAB isolates stems from their capacity to produce various inhibitory compounds, including lactic acid, acetic acid, hydrogen peroxide, and bacteriocins[26]. While the production of organic acids and hydrogen peroxide is typically controlled by chromosomal genes, bacteriocins and diacetyl are synthesized by plasmid genes[27]. The high concentration of lactic acid, a product of carbohydrate metabolism during fermentation, is a key antibacterial agent. Lactic acid suppresses *E. coli* growth by reducing the intracellular pH of the bacterial cells, which in turn damages the cell wall and disrupts its permeability. This disruption ultimately leads to cell lysis and death[28].

Beyond organic acids, bacteriocins also contribute significantly to antibacterial activity. These protein-like toxins inhibit the growth of competing bacteria by disrupting their energy production and protein biosynthesis. The size of the inhibition zone formed by bacteriocins is influenced by the target bacteria's cell wall characteristics, as structural differences can affect their susceptibility to these antimicrobial agents. The production of bacteriocins by probiotic bacteria is also modulated by environmental factors such as temperature, pH, carbon and nitrogen sources, and salinity of the growth medium[29].

Another important antimicrobial compound is hydrogen peroxide, which exerts its bactericidal effects by oxidizing the sulfhydryl groups of cellular proteins, leading to enzyme denaturation. Additionally, it causes peroxidation, an oxidation of lipid membranes that increases cell permeability. Finally, carbon dioxide, also produced during fermentation, can act as an antibacterial agent by inhibiting enzymatic decarboxylation[30].

CONCLUSION

This study successfully isolated three LAB strains from traditionally salt-fermented mandai vinegar and four strains from vinegar fermented with the *L. casei* strain Shirota starter culture. All of these isolates were confirmed to be Gram-positive, a key characteristic of LAB. Notably, isolate CML 1, which was derived from the starter culture fermentation, exhibited the most potent antibacterial activity against *E. coli*. These findings provide compelling evidence that incorporating a starter culture into the fermentation of mandai vinegar not only yields a higher number of viable LAB strains but also enhances their functional properties, specifically their antibacterial potential.

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AUTHORS' CONTRIBUTION

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Resources: Sapri, Rezia Elok Canrika
Software: Sapri, Rezia Elok Canrika
Supervision: Indah Woro Utami, Nishia Waya Meray
Validation: Indah Woro Utami, Sapri, Nishia Waya Meray
Visualization: -
Writing - original draft: Sapri, Nishia Waya Meray, Rezia Elok Canrika
Writing - review & editing: Indah Woro Utami

DATA AVAILABILITY

None.

CONFLICT OF INTEREST

The authors declare no conflicts of interest related to this study.

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