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Identification of Probiotic Potential *Lactobacillus* from *Mandai* using Biochemical Test

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Abstract

In this research, only *Lactobacillus* species originating from *mandai* were evaluated for their probiotic characteristics to determine their usability as functional ingredients in chempedak inner skin fermentation. *Mandai,* a traditional fermented food from chempedak (*Artocarpus integer*), holds potential as probiotic source yet limited research exists on its beneficial microbes, particularly *Lactobacillus* species. This study analysed *mandai* fermentation over 28 days revealing a peak lactic acid bacteria (LAB) count 2.75 x 10^4 CFU/g on day 7, followed by a decline to 1.40 x 10^4 CFU/g, indicating optimal conditions for probiotic viability early in fermentation. Gram-staining confirmed the presence of Gram-positive, rod-shaped *Lactobacillus.* These findings suggest *mandai's* potential as a natural probiotic, supporting the functional food development with further research needed for molecular characterization and in vivo validation.

1. Introduction

Lactic acid bacteria (LAB) are a group of fastidious, non-sporulating, and catalase-negative gram-positive microorganisms with an ability to grow in an environment with very low pH. These non-respiring rods or cocci lack cytochromes, and their distinctive metabolic and physiological characteristics relate to their ability to produce lactic acid as the primary product in fermentation. This lactic acid production not only supports their role in food preservation and flavour enhancement but also emphasizes their importance in various biotechnological applications. Studying lactic acid bacteria (LAB) is essential due to their profound impact on health, food technology, and biotechnology.

In Indonesia, there is a traditional fermented food called as *mandai* which are popular in Central South and East Kalimantan. *Mandai* is a traditional food that was prepared by using the inner skin of chempedak fruit (*Artocarpus integer*) [1]. According to [2] the fermentation process of *mandai* can occur randomly or be initiated by the addition of a starter culture. Lactic acid bacteria (LAB) that usually appear in this fermentation process are *Lactobacillus plantarum* and *Pediococcus pentosaceus.*

LAB is an important microorganism that produces lactic acid during metabolic activities. It plays many roles in agriculture, food and clinical industries [3]. LAB is used in many fermented foods as a food preservative which is being recognized and approved by the food industry. As LAB is crucial in many food applications, the food industry is always trying to find strains with good quality characteristics and properties to boost the food taste and longevity. LAB also has therapeutic properties that are crucial for human health benefits [3].

However, limited research has been conducted on the specific probiotic potential of *Lactobacillus* in *mandai*. This study aims to address this gap by identifying and characterizing *Lactobacillus* species from *mandai* through biochemical test. This research seeks to determine the presence of LAB when fermented at different fermentation

of time and to evaluating their morphological and probiotic characteristics that contributing to the development of functional food applications.

2. Methodology

2.1 Preparation of Samples of *Mandai*

Pulp samples of *mandai* were procured from suppliers located in the vicinity of Pagoh. According to Rahmadi et al., (2018), he reported that the outer skin of chempedak (*Artocarpus integer*) was taken. The outer and inner skin were removed leaving only the husk and the meat. The inner skin was then sliced thinly into pieces that were 2-3 cm across for the chempedak used for the experiment. After that, it was treated with distilled water at 100°C for 5 minutes to resize the sap from the skin. After that, the water was expressed out and reheated once more in a hermetically sealed procedure for 5 minutes at 100°C to enhance the texture. Following heat treatment, the temperature of the sample was reduced to a value below 40°C. *Mandai* did not even have any form of inoculation and spoiled naturally. The *mandai* was kept at a temperature of 27°C so as to ensure slow fermentation of the product. From the incubation phase onwards the *mandai* was drained by blending and the puree of *mandai* was dried for 18 hours at 45° C before it was grounded and sieved through an 80 mesh for composting. This was done for day 7, 14, 21 and 28 of fermentation time.

2.2 Isolation of LAB From M*andai* Powder

2.2.1 Preparation of Nutrient Agar

A total of 10 g of nutrient agar was dispersed in 500 mL of distilled water under stirring to avoid the formation of lumps. The solution was then heated in an autoclave at 121°C for 15 minutes so as to remove microbial load. After autoclaving, the nutrient agar solution was aseptically incorporated with sterile petri dishes all under a biosafety cabinet. The nutrient agar was set after a short resting time: It was then ready for subsequent use.

2.2.2 Spread Plating of Diluted M*andai's S*ample Into Nutrient Agar

One gram of each of *mandai's* samples that was taken at days 0, 7, 14, 21 and 28 was mixed with 10 mL of sterile distilled water. After that, the mixture was allowed to stand for 15 minutes so that the particle could completely disperse and dissolve in the liquids and make it a homogeneous mixture. Thereafter and in compliance to the test procedure, 0.1 mL of the diluted *mandai's* sample was aspirated aseptically using micropipette and spread on a sterile petri dish containing solidified nutrient agar. The plates were then incubated at 27°C for 24 hours to allow the bacteria to grow.

2.2.3 Inoculation of Bacteria on Nutrient Agar

When there was bacterial growth on the petri dish then aseptic technique was used to transfer bacterial growth from the nutrient agar plate to fresh nutrient agar plates using an inoculation loop. Inoculation loop was put through flame to control the transfer of bacteria which was involved in the isolation process in order to control contamination. The plates that were separately had their antibacterial agent, the inoculated plates were incubated at 27°C for 24 hours for bacterial growth [5].

2.3 Morphological Characteristics of Selected LAB Isolates

The isolated lactic acid bacteria LAB were further characterized by the gram staining technique to differentiate the cell wall structure of LAB according to their morphological and biochemical property.

2.3.1 Gram Staining

Firstly, a slide smear containing a single colony of LAB was prepared. A single colony of LAB was added to the slide using a sterilized inoculation loop. A drop of water was added to the slide to mix with the colony on the slide. Then, the slide was moved over a gentle flame in a circular movement for a few seconds until the mix colony with water was fully dried.

Subsequently, spread the smear on the petri dish and stain the layer of the sample with crystal violet dye for 60 seconds before washing it with water. The iodine solution was added for 60 seconds to fix the stain and then decolorized using ethanol and followed with another water rinse. Afterward, Safranin solution was used as the counterstain for 30 seconds and then washed off. The slide was allowed to air-dry and then studied under the microscope in order to effectivity of LAB cells.

The slide was observed under a light compound microscope using X40 until the X100 objective lens. Immersion oil was used at the X100 objective lens. The morphology of the LAB was observed.

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3. Result and Discussion

3.1 3.1 Introduction

Biochemical test were chosen over other methods because they effectively identify and characterize *Lactobacillus* species based on their metabolic traits, such as acid-bile tolerance, antibiotic susceptibility, and antimicrobial activity which are essential for probiotic evaluation. Compared to molecular techniques like PCR, biochemical tests are more cost-effective, accessible, and practical for assessing probiotic functionality in food fermentation. They provide direct insights into fermentation related properties, ensuring the usability and viability of *Lactobacillus* as functional ingredients in *mandai* fermentation for potential health applications. The study utilized triplicate samples for each fermentation time interval which are days 0,7,14,21 and 28 to ensure accuracy and reliability of bacterial count and probiotic characteristics assessments.

3.2 3.2 Fermentation of M*andai*

When *mandai* was fermenting, lactic acid was shown to be the main end product which was formed during the fermentation process. Lactic acid produced through the metabolism of LAB was responsible for making the environment acidic.

Fig. 1 mandai fermentation in an air tight container

The changes in the colour, texture, shape and odour of the *mandai* from day 0 to day 28 were noted thereafter. At first, the *mandai* pieces looked fluffy, very tender and slightly firm and wet, this was expected given that the pieces had been boiled then fermented. Cross-sectional texture change was observed over fermentation period where the texture became softer though having slightly disintegrated due to increased microbial activity and enzymatic action during fermentation.

The odour profile of the *mandai* also varied with the state of fermentation. Initially, it had a relatively sweet smell, which most probably resembled that of chempedak's flavour profile. With increased fermentation, the smell beamed a more complex and typical LAB smell of being either acidic or sour. By days 21, the off-thread odour became pronounced due to the synthesis of organic acids and other substances formed in the course of the fermentation process.

3.3 Lactic Acid Bacteria (LAB) Present at Different Fermentation Time

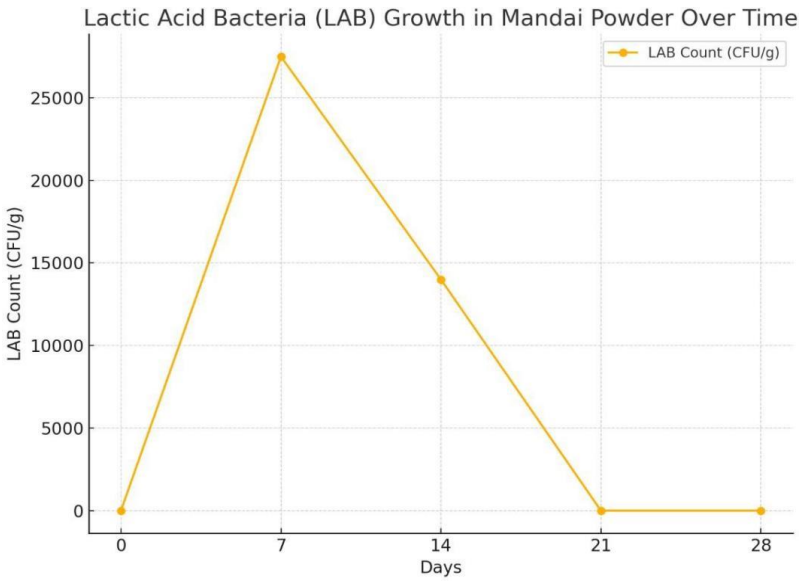

Fig. 2 Graph of Lactic Acid Bacteria (LAB) growth in *mandai* powder over time

Fig. 2 illustrates the growth dynamics of Lactic acid bacteria (LAB) in *mandai* powder over 28 days. At the start (Day 0), LAB was not detected which might be either cause by the absence of active bacterial colonies or that the bacteria were in a dormant state. This could be attributed to the initial conditions of the sample, where nutrients or other factors were not yet conducive for bacterial proliferation.

LAB count raised substantially on the 7th day with 2.75 x 10^4 CFU/g. That peak shows that *mandai* powder enhances bacterial growth which can be useful in uses such as in the production of probiotics or in fermentation processes. A decline starts after Day 7 and LAB count reduced to 1.40 x 10^4 CFU/g. This may be attributed to nutrient limitation, build-up of inhibitory substances such as lactic acid that silence bacterial metabolic activity, or unfavourable environmental factors that compromise bacterial viability. By Days 21 and 28 there was presence of LAB but the count was zero, this could mean the environment had become hostile to the growth of LAB by factors such as resource depletion or the surroundings had changed dramatically for instance in pH level.

The findings point to Day 7 as the right time window within which LABs should be active in powder samples originating from *mandai*. The decline beyond this point indicates that further enhancements are required to support the LAB stability; the measures such as optimal storage condition or encapsulation.

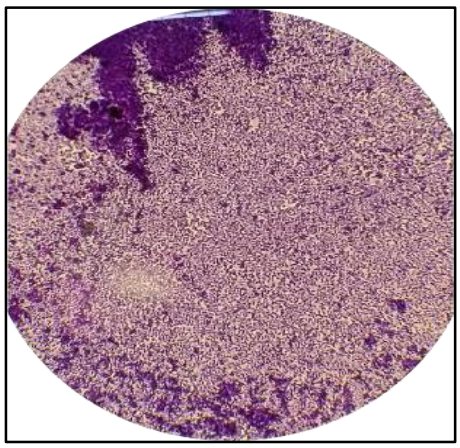

Fig. 3 The growth of LAB on nutrient agar

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Fig. 4 Gram stain of selected LAB

3.4 Morphological Characteristics of LAB From *Mandai's S*ample

In this study, Lactic Acid Bacteria (LAB) were isolated from a sample of *mandai*. The isolation was accomplished by following a routine microbiological process and inoculation on a solid medium. After inoculation of appropriate dilutions of *mandai's* sample on nutrient agar, successfully cultured LAB isolates were subjected to integrated phenotypic identification which includes morphological, and biochemical tests. Fig. 3 shows the growth of isolated LAB on nutrient agar.

Fig. 3 shows the morphology of the LAB on nutrient agar, which has a yellowish-white colour with a circular-shaped colony and glistering-convex elevation. The LAB isolation will produce a colony with a yellowish-white colour in MRS agar, a clear zone around with a size around 0.5- 2mm, circular shape, and non-fibrous. [6] also stated that LAB isolates have round shapes, white or creamy colours, smooth margins, and clear zones around their colony.

In Fig. 4 of this study, it shows a light microscope view of LAB at 100x magnification after gram-staining the bacteria to reveal some of their features. LAB was reported to be morphologically rod-shaped (bacilli) and observed to be Gram-positive, having a purple colouration. [5] define Gram-positive bacteria as those with a thick cell wall, measuring 20-80μm in thickness and retaining purple or blue colour in the gram stain. On the other hand, Gram-negative bacterium has a thin cell wall of less than 10 μm, an outer membrane containing pores and appendices, so they are coloured pink or red.

Microscopically LAB are Gram-positive and are found to have single, pairs, or short chains [3]. Gram staining is important for differentiating bacteria into Gram-positive and Gram-negative species, based on the ability to take up crystal violet when treated with safranin. LAB was characterized as Gram-positive, rod-shaped (bacilli), and purple-coloured on days 7 and 14 of observation.

This observation is in agreement that stated that the LAB group of microorganisms are defined as Gram-positive, rod-shaped or circular-shaped, facultatively anaerobic, non-spore-forming, and form lactic acid as the main product from carbohydrate fermentation and further elaborated that upon washing with alcohol and red safranin staining, Gram-positive bacteria remain purple in colour, while Gram-negative bacteria turn red.

Previous studies have also reported the morphological characteristics of LAB produced during milk fermentation observed that LAB isolated are Gram-positive, bacilli, and non-spore-forming.

The specific staining of the microorganism's purple under the gram stain method indicates that they are Gram-positive, which is a typical feature of LAB associated with the thickness of peptidoglycan cell wall. This high bacterial density is indicative of targeting and consistent with peak LAB activity usually seen during the fermentation phase (i.e., Day 7 in this study). Their behaviour at lower pH values and salt (NaCl) levels also reinforces their importance in fermentation. These findings strongly suggest that the microorganisms in the image are LAB, contributing significantly to the production of lactic acid and other beneficial by-products essential for the fermentation process.

When compared to other fermented foods, LAB from dairy-based products like yogurt and kimchi have been extensively studied for their probiotic benefits. Studies on fermented milk have demonstrated LAB's ability to enhance gut health and inhibit pathogenic bacteria, a trait similarly observed in *mandai* fermentation. Additionally, research on fermented vegetables shows LAB strains that can survive acidic environments and contribute to bio-preservation, aligning with this study's findings on LAB stability in *mandai*.

These results emphasize *mandai's* potential as a novel functional food ingredient, offering an alternative to conventional probiotic sources. Future studies should focus on molecular characterization and in vivo probiotic efficacy to validate its health benefits and commercial applications.

4. Conclusion

Biochemical characterization and analysis of the *Lactobacillus* species isolated from *mandai* was successfully achieved by this study. Lactic Acid Bacteria (LAB) activity was highest at the early stages of fermentation (Day 7) indicated by the highest colony-forming units and optimum morphological and biochemical traits.

The study additionally highlighted the significance of fermentation period, although the viability and activity of LAB were mixed, a decrease in bacterial count was recorded post-peak period. This indicates that the viability of probiotics needs to be preserved for functional food applications, which would require strategic fermentation conditions or storage methods. The research advances our understanding of the probiotic properties inherent in *mandai* and opens pathways for its use in developing health-promoting products.

A key limitation of this study is the reliance on biochemical characterization without molecular identification, which could provide more precise taxonomic classification of *Lactobacillus* species in *mandai*. Additionally, the study focused on in vitro assessments of probiotic potential and further in vivo studies are needed to confirm the health benefits in human or animal models. Future research should explore genomic sequencing for strain-level identification, investigate the long-term stability of LAB in functional food applications, and assess the effects of *mandai*-derived probiotics on gut microbiota and immune responses to validate their potential for commercial probiotic development.

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Conflict of Interest

The authors confirm that there are no conflicts of interest related to the publication of this paper.

Author Contribution

The authors confirm contribution to the paper as follows: ***study conception and design:*** *Nur Zaidatul Ilya Hussain;* ***solve the governing equation:*** *Nur Zaidatul Ilya Hussain, Siti Fatimah Sabran;* ***data collection:*** *Nur Zaidatul Ilya Hussain;* ***analysis and interpretation of results:*** *Nur Zaidatul Ilya Hussain, Siti Fatimah Sabran;* ***draft manuscript preparation:*** *Nur Zaidatul Ilya Hussain, Siti Fatimah Sabran. All authors reviewed the results and approved the final version of the manuscript.*

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