Screening Lactic Acid Bacteria from Seaweed for Plant Milk Fermentation: A Preliminary Research

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Abstract. A fascinating opportunity for screening lactic acid bacteria with the potential to ferment plant-based milk production exists in seaweed, a rich source of microbial variety. In this work, marine-derived lactic acid bacteria (LAB) will be identified and used to improve plant milk fermentation, paving the path for healthy and sustainable dairy substitutes. The methods applied in this research including the LAB isolation and identification by performing gram and catalase test. Then, the lactic acid bacteria transferred to fermentation in both plant and dairy milk to observe the ability to ferment plant-based products. It is resulted that the bacteria isolated can ferment the plant-based milk better than the dairy milk, suggesting that the potential of marine lactic acid bacteria to be applied in plant milk-based fermentation.

1 Introduction

The contemporary food industry is witnessing a notable trend with the increasing development of plant-based food and beverage products. Among these products is plant-based milk, exemplified by soy milk, which not only offers a high nutritional content beneficial for health but also serves as an alternative for vegetarian individuals and those with lactose intolerances. As the focus on the health benefits of plant-based milk intensifies, fermentation processes have gained prominence for augmenting its nutritional profile [1]. This shift towards plant-based alternatives is not solely motivated by health considerations; it is also propelled by the understanding that traditional dairy production is considerably less sustainable due to high land and water use [2]. Hence, there is a growing imperative to transition towards more sustainable options.

Transitioning from this sustainability perspective, fermentation processes become crucial in optimizing the nutritional content of plant-based products [3]–[6]. Fermentation, a chemical process executed by microorganisms through enzymatic activities, plays a pivotal role in breaking down complex organic molecules into simpler forms. This process not only enhances the nutritional content of food but also contributes to improved flavor and appearance [7], [8]. Key influencers of fermented foods include the composition of the

substrate and the microorganisms involved in the fermentation process, with a notable emphasis on lactic acid bacteria (LAB).

Lactic acid bacteria constitute a group of gram-positive bacteria lacking spores, exhibiting spherical or rod-shaped forms, and demonstrating the ability to convert carbohydrates into lactic acid. This group of microorganisms encompasses types that are not only harmless but also beneficial for health [9]. Their utility extends to the production of functional foods, exemplified by probiotic drinks known for their digestive health benefits. The fermentation process carried out by lactic acid bacteria in probiotic drinks involves the hydrolysis of lactose into acid. While existing probiotic beverage products predominantly utilize dairy, this research seeks to explore the isolation of lactic acid bacteria from seaweed, aiming to uncover the potential of seaweed as a source of lactic acid bacteria and assess the capability of the isolated strains in fermenting both plant and dairy milk.

2 Material and Method

2.1 Materials

Materials used in this study, namely *lawi-lawi*, soymilk (Naraya), UHT milk (Ultra Milk), de Man Rugosa Sharpe Agar (MRSA, Merck), de Man Rugosa Sharpe Broth (MRSB, Himedia), Nutrient Agar (NA, Merck), calcium carbonate ($CaCO_3$, Pudak), NaOH 0. 1 N (Merck), NaCl (Merck), 1% phenolphthalein (PP) indicator (ROVA), crystal violet solution (Merck), safranin solution (CDH), lugol solution, Hydrogen Peroxide (H_2O_2), distilled water, alcohol 75%.

2.2 Method

2.2.1 Research Design

This study applied three stages namely lactic acid bacteria isolation, identification (through visual observation, gram staining, and catalase test), and application in drink fermentation. In fermentation two factors were applied; lactic acid bacteria addition (with and without the addition of lactic acid bacteria) and type of drink (plant and dairy product). The fermentation carried out with three replications for each combination treatment.

2.2.2 Sample Preparation

The incubation process refers to the method [10]. *Lawi-lawi* used for incubation was obtained from Laikang village, Mangarabombang sub-district, Takalar Regency, then brought to the Laboratory for incubation. The seaweed incubation sampling process begins with 10 g of *lawi-lawi* separated, then washed thoroughly using tap water. After cleaning, the *lawi-lawi* was rinsed again using distilled water, then the sample was incubated for seven days at 25°C.

2.2.3 Lactic Acid Bacteria (LAB) Isolation Preparation

The isolation process of lactic acid bacteria was performed by diluting the fermented seaweed in 9 mL of peptone water and then vortexed. The serial dilution then performed to 10^{-7} concentration. Then 0.1 mL of each dilution was inoculated on MRSA + 1% CaCO media with the spread plate method and then incubated in an incubator for 48 hours at 37°C. The

colony with clear zone indicating the production of acid then tranferred to new media for further purification and identification.

2.2.4 Lactic Acid Bacteria Identification

The identification of lactic acid bacteria was performed through visual observation of the colony, gram staining, and catalase test.

For the visual observation the colonies that had been purified on petri dishes was then observed visually. The Lactic Acid Bacteria was freshly inoculated into a new culture media and incubated for 48 hours prior to observation on shape, edge, elevation, and color.

Gram staining is carried out following [11] with a light modification in fixation in which in this study heat fixation using flame was applied. The cell shape and color are observed under microscope with 1000x magnification, if red indicates that the bacteria are gram-positive while purple indicate s that the bacteria are gram-negative.

The catalase test is performed to determine the presence of the catalase enzyme in bacterial organisms, which serves as a catalyst for the breakdown of hydrogen peroxide (H_2O_2). This test involves aseptically obtaining a sample from a single bacterial colony and depositing it onto a object glass previously sterilized with 70% alcohol. Subsequently, the substrate is treated with a 3% hydrogen peroxide solution, and the formation of observable air bubbles is noted. The presence of bubbles signifies a positive result, indicating the bacterium's possession of the catalase enzyme, whereas the absence of bubble formation indicates a negative outcome.

The isolated processed to fermentation only those who meet the criteria suspected lactic acid bacteria namely gram positive and catalase negative.

2.2.5 Starter Preparation

Starter preparation refers to [12] with modifications. One colony were inoculated on MRSB media and subsequently incubated for 48 hours at 37°C. The suspension contains lactic acid bacteria were then placed into a sterile 4 ml Eppendorf tube, which was then subjected to centrifugation at 5000 rpm for 15 minutes to facilitate the separation of cell masses. The obtained cell mass was subjected to two rounds of washing using a normal saline solution (NaCl 0.85%) to eliminate any residues from the media. Following the washing process, the mass then suspended with the saline solution and the absorbance was measured at a wavelength of 600 nm, set at 1.2.

2.2.6 Fermentation

The milk fermentation process refers to [13] with modifications. Milk (plant and dairy) were put into sterilized glass bottles with a volume of 140 mL each. Each treatment was inoculated with 5% bacterial starter, then the milk was incubated at 37^{o}C for 48 hours.

2.2.7 pH

The pH test was conducted with a PHS-3C pH meter [14]. Prior to the meassurement, the pH meter was calibrated then the samples were measured by inserting the stick into the samples. The measurement results are displayed on the screen.

2.2.8 Total Acid

Total acid content analysed using titration method [15]. The sample was taken 10 mL and put into an erlenmeyer flask to be titrated with 0.1 N NaOH. The indicator used is phenolptalein 1% with a color change from colorless to pink. Acid content is calculated using the formula:

$$\text{Acid Content} = \frac{V1\ x\ N\ x\ BM}{V2\ x\ 1000} \text{ x } fp \text{ x } 100$$

Description: V1 = NaOH volume (ml), V2 = Beverage volume (ml), N = Normality of NaOH (0.1), BM = Molecular Weight (90), and fp = Dilution Factor (100/25).

3 Result and Discussion

3.1 Morphological characteristics

The morphological analysis of lactic acid bacteria involves identifying them by observing the shape, color, edges, and elevation of bacterial colonies. It is expected that the bacterial isolates obtained have a circular shape, white or cream color, raised or convex elevation. The results of isolates observations can be seen in table 1.

Table 1. Morphological characteristics of isolated microorganisms.

Isolate Code	Form	Margin	Elevation	Color
A	Circular	Entired	Raised	Cream
C	Circular	Entired	Raised	Cream
D	Circular	Entired	Raised	Cream
E	Circular	Entired	Raised	White

Based on the table 1, it is evident that all isolates exhibit a consistent morphology characterized by a circular or rounded shape with smooth edges. The margin uniformly displays an entire or evenly shaped colony, while the colony elevation also shares a consistent trait of being raised, providing distinct height across the entire surface. In terms of color, isolates with codes A, B, and C share a similar cream hue, whereas code E exhibits a white coloration. These characteristics collectively align with the typical morphology of lactic acid bacteria [16]. Thus, isolates continue for microscopic shape, gram staining, and catalase test.

3.2 Microscopic shape, Gram Staining and Catalase

Lactic acid bacteria typically exhibit round or rod-shaped cells and fall within the category of Gram-positive bacteria, appearing purple when examined under a microscope. In the catalase test, these bacteria show a negative result as they do not produce gas bubbles. This absence of gas bubbles is attributed to the presence of the catalase enzyme, which effectively

converts hydrogen peroxide into water and oxygen. Therefore, lactic acid bacteria are characterized as catalase-negative [17].

In this study, microscopic morphological analysis was performed complemented with gram staining and catalase analysis. It is anticipated that bacteria isolated from seaweed will exhibit either round or rod-shaped cells, fall into the category of Gram-positive bacteria, and display negative catalase activity. Detailed results of the microscopic morphology analysis, Gram staining, and catalase test for the isolates are presented in Table 2.

Table 2. Microscopic shape, Gram Test and Catalase of isolated microorganisms.

Isolate	Isolate Characteristic		
	Shape	Gram Test	Catalase Test
A	Basil	Positive	Negative
C	Basil	Positive	Negative
D	Tetracoccus	Positive	Negative
E	Coccus	Negative	Positive

The table reveals that microscopic observations of the isolates show shapes resembling bacilli, cocci, and tetra cocci, suggesting that all isolates could potentially be considered as lactic acid bacteria (LAB) based on their morphological characteristics. However, upon conducting gram staining and catalase tests, it becomes evident that only isolates A, C, and D exhibit traits consistent with lactic acid bacteria. Specifically, they display a gram-positive nature and a negative catalase reaction. Consequently, only these isolates are identified as lactic acid bacteria and are deemed suitable for further application in the fermentation process.

3.3 pH Value

pH serves as an indicator of the acidity or alkalinity of a solution. In this study, pH testing was employed to ascertain the acidity of a solution. The measurement of pH was specifically conducted to evaluate the reduction in pH resulting from the production of acids by lactic acid bacteria within the product. Based on [18], the pH value in dairy milk and soybean juice decreased during the fermentation process and a similar decrease was expected in this study. The results of pH measurements on dairy milk and soy milk are presented in Figure 1 and 2.

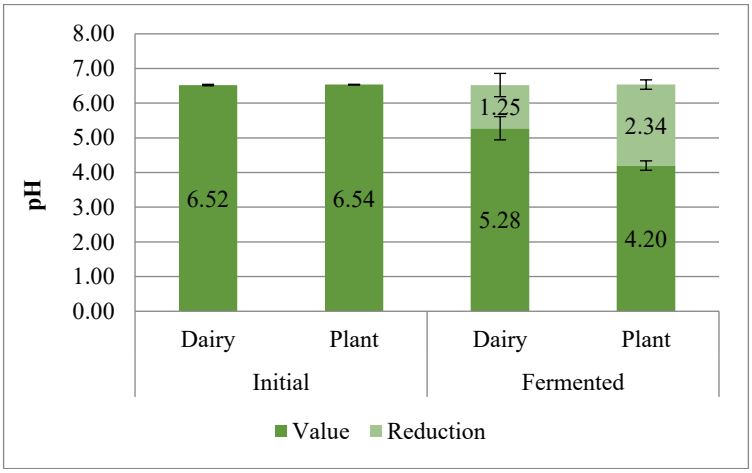

Fig. 1. pH value before and after fermentation with different isolates.
Notes: Values shown are the average of three replicates of each treatment.

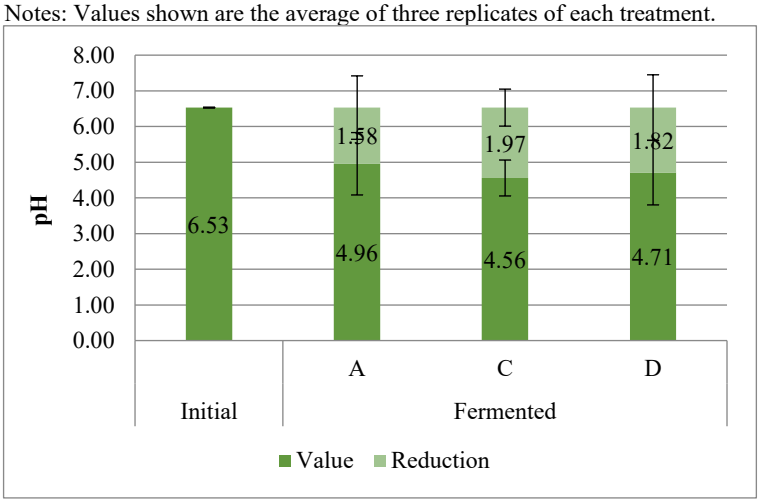

Fig. 2. pH value before and after fermentation of plant milk and Dairy milk.
Notes: Values shown are the average of 3 replicates for each treatment.

Figure 1 illustrates that the initial pH of both dairy and plant milk was approximately 6.5. Following fermentation, both experienced a decrease in pH, with plant milk exhibiting a higher reduction (2.34) to reach a final pH of 4.20. This outcome suggests that lactic acid bacteria are more effective at fermenting sugars in plant milk compared to dairy milk, resulting in the production of organic acids. It is hypothesized that the lactic acid bacteria, isolated from the plant product (seaweed), may possess an increased proficiency in fermenting plant-based substances, as they are adapted to that specific environment.

Figure 2 displays the average pH reduction for each isolate during the fermentation of both types of milk. Notably, all isolates, suspected to be lactic acid bacteria, demonstrate a decrease in pH, with isolate C exhibiting the highest reduction. Further experiments are essential to identify and characterize these bacteria before their application in subsequent research can be considered.

3.4 Total Acid

Total acid, reflecting the amount of acid in a solution, was analyzed in this study to observe the increase in acidity resulting from lactic acid bacteria fermentation. It was expected that lactic acid bacteria could efficiently ferment both dairy milk and plant milk, leading to a rise in the total acid content. The specific results of the total acid measurements for dairy milk and plant milk are presented in Figure 3 and Figure 4.

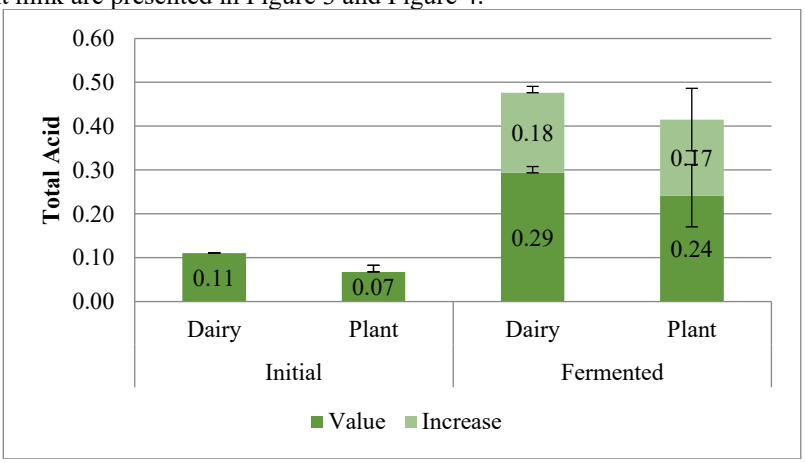

Fig. 3. Total Acid Value Before and After Fermentation with Different Isolates.
Notes: Values shown are the average of 3 replicates for each treatment.

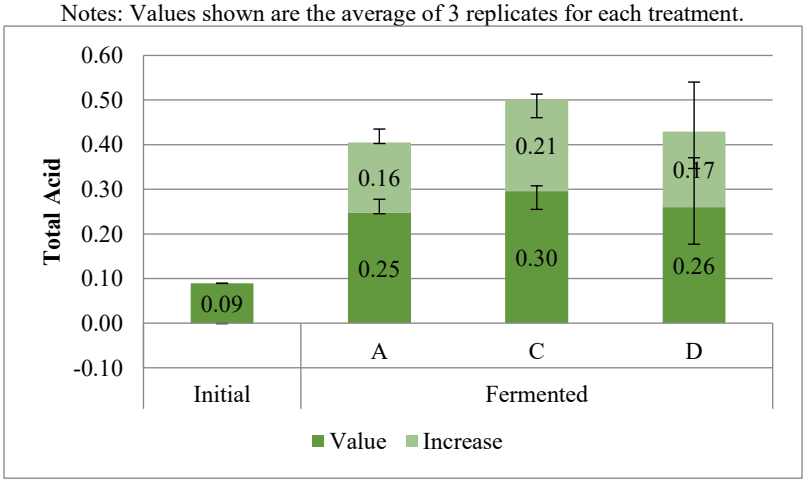

Fig. 4. Total Acid Value Before and After Fermentation of Plant Milk and Dairy Milk.
Notes: Values shown are the average of 3 replicates for each treatment.

Figures 3 illustrate the elevation in total acid levels in both dairy milk and plant milk following fermentation, accompanied by a decrease in pH values (Figure 1). Throughout the fermentation process, lactic acid bacteria (LAB) generate lactic acid by metabolizing carbohydrates and other organic components present in the substrate [19]. The lactic acid content plays a significant role in contributing to the overall acidity of fermented products, leading to an increase in the total acid levels in both plant and dairy milk after fermentation. It's intriguing to observe in Figure 4 that isolate C exhibits the highest increase in total acids, aligning with the observed highest reduction in pH (Figure 2).

4 Conclusions

The findings indicate that seaweed serves as a viable source of lactic acid bacteria. The lactic acid bacteria isolated from seaweed exhibit proficiency in fermenting both plant and dairy milk, displaying enhanced performance particularly in plant-based milk products.

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