

## COMPARISON OF LACTIC ACID BACTERIAL ISOLATES FROM VARIATIONS IN TIME FERMENTATION PROCESS OF CEMPEDAK SKIN

Syifa Noorwina, Siti Nashihah \*

Department S1 Pharmacy, Faculty of Pharmacy, University of Muhammadiyah Banjarmasin, Indonesia

\*Email: siti.nashihah@umbjm.ac.id

### ABSTRACT

*Cempedak is a tropical native fruit from Indonesia and can be consumed by fermenting the skin fruit using a salt solution. The results of the fermentation called Mandai. Fermentation will be successful if there is a role of Lactic Acid Bacteria (LAB). Therefore, this study was conducted to determine the content of LAB in Mandai and isolates when fermented at different times. This study was carried out by dilution and the Pour Plate method, identifications such as morphological identification by gram staining and biochemical test by catalase t and TSIA test. Identification of this study was the observation of clear zone around the bacteria grown on MRSA media as a selective medium for LAB plus CaCO<sub>3</sub>. In this study, 5 samples of Mandai were used with different fermentation times. The results of the LAB isolation study in Mandai showed that the longer the Mandai fermentation was carried out, the more colonies there would be. While the LAB type was only found in sample 5 which was fermented within 20 weeks. The results obtained were following the characteristics of LAB, including gram-positive bacteria in the form of bacilli or cocci, not having catalase enzyme, and being able to ferment carbohydrates.*

**Keywords:** Mandai, Traditional food, Fermentation, Lactic Acid Bacteria (LAB)

### INTRODUCTION

Cempedak (*Artocarpus integer*) is a tropical fruit plant native to Indonesia that is spread in tropical and subtropical areas, especially in Southeast Asia such as Indonesia<sup>1</sup>. Based on data from the Central Statistics Agency (BPS) it is stated that cempedak fruit production in Indonesia reached 775,480 tons in 2018, 779,859 tons in 2019, and 824,058 tons in 2020. Based on data from the Central Statistics Agency (BPS) it is stated that

the production of cempedak fruit in 2020 in West Kalimantan, Central Kalimantan, South Kalimantan, East Kalimantan, and North Kalimantan reached 11,612 tons, 13,652 tons, 20,375 tons, 13,584 tons, and 8,254 tons<sup>2</sup>.

In the Kalimantan area, the flesh of ripe cempedak fruit skin is used as an additional food by fermentation, namely soaking in salt for several days on the part of the fruit than is usually

called Mandai<sup>3</sup>. Mandai is a fermented food typical of South Kalimantan and East Kalimantan which is traditionally made

from the inner skin of the cempedak fruit that has been cleaned and soaked in a salt solution<sup>3</sup>. Fermentation of the flesh of this cempedak fruit peel varies from 3 days to more than 30 days depending on the tastes of the individual who processes it. The success of this fermentation process is very dependent on the use of Lactic Acid Bacteria (LAB) because these bacteria are microorganisms that are commonly used in the fermentation process with safe metabolic activities and utilize available sugars to produce organic acids and other metabolites<sup>4</sup>.

Rahayu (2003) has conducted research on microorganisms involved in Mandai fermented food and has succeeded in isolating 9 bacteria, two of which are *Lactobacillus plantarum* and *Pediococcus pentosaceus*. Both of these bacteria belong to the LAB group<sup>3,5</sup>. These bacteria have been characterized as probiotics or live microorganisms which if consumed in sufficient

quantities will provide health benefits for those who consume them<sup>6</sup>. Lactic Acid Bacteria (LAB) are members of a heterogeneous group of bacteria that play an important role in various fermentation processes. Some foods that can be fermented and produce these bacteria are fermented milk, meat, fish, fruit, vegetables, and cereal products. One of the functions of these bacteria is to produce lactic acid in food acidification and these bacteria can contribute to the taste, aroma, texture, and nutritional value of food and can produce a therapeutic effect so that they can become probiotics<sup>4</sup>.

Emmawati, et al (2015) isolated LAB from Mandai in a 12-day fermentation process showing the production of more metabolites, including organic acids, thereby lowering the pH and making the atmosphere acidic in the range of 4.16-4.8 with total bacteria [3]. 5 log CFU/ml at the start of fermentation, 6 log CFU/ml on day 5 and 7 log cfu/ml on day 14. Meanwhile, Siregar et al (2014) isolated LAB from Mandai in a 14-day fermentation process with more emphasis on decreasing the pH of

Mandai and growing bacterial isolates, where the decrease in pH of Mandai reached 3.83 and showed that the bacterial isolates obtained consisted of 9 Gram-negative isolates were obtained on day 0 to day 4 and 8 Gram-positive isolates were obtained on day 6 to day 14<sup>7</sup>.

In addition, Mandai fermentation carried out by the community usually takes more than 14 days. Therefore, this study was conducted to determine the presence of Lactic Acid Bacteria (LAB) which plays a role in the fermentation process, the characteristics of these bacteria, to find out the differences in isolates from Mandai which have been made with different fermentation times, both from 7 days and more than 30 days and the total number of LAB from the sample used. The methods used in this study were the collection of Mandai samples with different fermentation times, isolation of bacteria with MRS medium, characterization of bacterial morphology, biochemical tests of bacterial isolates, and Total Plate Count (TPC) tests.

## **METHOD**

### **Material**

The materials used in this study were the mesocarp skin of cempedak fruit (*Artocarpus Integer*), distilled water, table salt, De Man Ragosa Sharpe Agar (MRSA) media, 0.9% NaCl, CaCO<sub>3</sub>, 95% ethanol, immersion oil, Gram A dye, Gram B, Gram C, Gram D, 3% H<sub>2</sub>O<sub>2</sub>, and TSIA media.

### **Sample Collection**

The main criteria for selecting the sample were that the Mandai was made from the flesh of the skin of the cempedak fruit (*Artocarpus integer*), had different fermentations from other samples and was fermented in a tightly closed state. This sample is obtained by making it yourself and collected from several sellers.

### **Isolation of Lactic Acid Bacteria**

#### **(LAB) in Mandai**

Isolation of LAB was carried out according to research from Siregar, et al (2014) with several modifications [7]. Preparation A total of 1 g of Mandai sample was taken from each sample, diluted in stages in 0.9% NaCl solution and homogenized using a

vortex for 30 seconds. The dilution was carried out in stages until a dilution of 10<sup>-6</sup>. A total of 0.1 ml of the sample solution from dilutions 10<sup>-5</sup> and 10<sup>-6</sup> was inoculated evenly on MRSA medium and incubated for 48 hours at room temperature. To differentiate acid-producing bacteria from other bacteria, 1% CaCO<sub>3</sub> was added to MRS agar. Colonies showing a clear zone around them were randomly selected as acid-producing bacteria.

### **Gram Staining**

Gram staining was carried out according to the rules of the gram staining protocol<sup>8</sup> and the gram staining guidelines<sup>9</sup>. Prepare preparations for glass objects for painting. Gram staining is done by making smears of bacterial isolates that have been made. Add about 5 drops of Hucker's Crystal violet paint solution for 1 minute. Excess dye is rinsed off with running water. Add about 5 drops of Mordan Lugol's Iodine and let sit for 30 seconds. Rinse with running water. Perform decolorization by flowing 95% ethanol on the smear from the tip of the slide at an angle. Stop when no more dye can be seen flowing from the slide. The maximum decolorization

time is 30 seconds depending on the thickness of the smear. Removes 95% ethanol by rinsing with running water. The isolate smear was then stained with Safranin solution for 20 seconds. Rinse again with running water and then dry. Tilt the slide to drain any remaining water on the smear and allow the slide to dry in free air. The morphological observations of the representative isolates were carried out by Gram staining where Gram-positive bacteria retain Crystal violet and safranin in the cell membrane and Gram-negative bacteria cannot maintain Crystal violet staining<sup>10</sup>. So, gram-positive cells will be colored purple and gram-negative cells will be colored pink<sup>8</sup>.

### **Catalase Test**

The catalase test was used to determine the presence of the catalase enzyme in bacterial isolation. Catalase is an enzyme that can catalyze the decomposition of hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) into air and O<sub>2</sub>. Lactic acid bacteria are a group of bacteria with negative catalase. The catalase test was started by taking one ose of isolate from MRS agar, then smeared it on an object glass that had been given alcohol. The

glass object was dripped with 3% H<sub>2</sub>O<sub>2</sub> solution and observed for the formation of gas bubbles on the preparation. Lactic acid bacteria are a group of bacteria with negative catalase which is characterized by the absence of gas bubbles<sup>11</sup>.

### **TSIA Test**

Triple Sugar Iron Agar (TSIA) test is a biochemical test carried out to determine the ability of bacteria to ferment carbohydrates such as glucose, lactose, and sucrose<sup>12</sup>. This test uses TSIA media containing glucose, lactose, and sucrose. The sterilized TSIA media is poured into a test tube then tilted until it is solid. There are two treatments on this medium, namely in the center of the inoculated bacteria on a straight needle by stabbing it until it reaches a depth of the media and on the sloping surface inoculating the bacteria by scratching. After being given these two treatments, the media was incubated for 1x24 hours at 37°C<sup>13</sup>. The TSIA test will show a positive result in the ability to ferment lactose and sucrose if a yellow color is formed on the medium, while a positive result in the ability to ferment glucose if a red or pink color is formed on the surface of the medium and yellow color

on the bottom of the medium and if the medium is cracked and lifted proves that there is the ability to produce gas as well as the formation of H<sub>2</sub>S gas if a black ring appears on the bottom of the media<sup>14</sup>.

## **RESULTS AND DISSCUSSION**

### **Sample Collection**

The main criteria for selecting the sample was that the Mandai was made from the flesh of the skin of the cempedak fruit (*Artocarpus integer*), had different fermentations from other samples and was fermented in a tightly closed state. Samples A, B, and C are samples of the results of the researchers' fermentation made by soaking the flesh of the skin of the cempedak fruit in a ship brand salt solution with fermentation times of 4 weeks, 6 weeks, and 8 weeks. Sample D is a sample obtained from a market in the Banjarmasin area which is fermented using kiloan salt solution for 12 weeks. Meanwhile, sample E is a sample of the result of immersion using dugong salt for 20 weeks which according to the local community, dugong salt is suitable for food

fermentation, this sample was obtained from traders in Batu Mandi District, Balangan Regency where the area is famous for many traders who sell Mandai almost along the road.

**Table 1. Types of salt in fermentation**

No	Code	Salt Type	Description
1	A	Cap Kapal	Hard
2	B	Cap Kapal	Hard
3	C	Cap Kapal	Hard
4	D	Kilos	Soft
5	E	Duyung	Soft

### Sample Dilution

Dilution is one way to reduce the number of bacterial colonies in the sample so as to facilitate the process of bacterial isolation<sup>15</sup>. The solution used in this dilution is a physiological saline solution or 0.9% NaCl. The solution was chosen to maintain the resistance of Lactic Acid Bacteria (LAB) because it is a sterile solution that can be used as a source of minerals that can meet the nutritional needs of microbes and can maintain an isotonic state of cells to avoid cell rupture from these microbes<sup>16</sup>. The sample used for the isolation of Lactic Acid Bacteria (LAB) was the result of dilutions of  $10^{-5}$  and  $10^{-6}$  in

order to obtain fewer bacterial isolates, making identification easier. Samples resulting from direct dilution are tested to avoid reducing sample quality and contamination from other microbes.

### Isolation of LAB in Mandai

Isolation was carried out on MRSA media with the addition of Calcium Carbonate ( $\text{CaCO}_3$ ) as one of the early stages of selection against Lactic Acid Bacteria (LAB). Calcium Carbonate ( $\text{CaCO}_3$ ) has alkaline properties that play a role in neutralizing lactic acid as a result of LAB<sup>17</sup>. Therefore, Lactic Acid Bacteria (LAB) will be indicated by the presence of a clear zone that appears around the colony. The clear zone appears as a form of the reaction of alkaline properties of  $\text{CaCO}_3$  in media with acidic properties of Lactic Acid Bacteria (LAB).

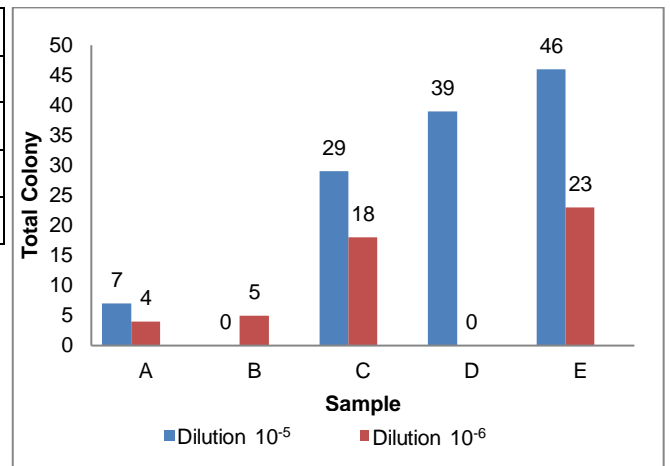
**Table 2. Isolate of Lactic Acid Bacteria (LAB)**

Code	Total (colony)	Clear Zone	Fungus Growth
A5	7	-	+
A6	4	-	+
B5	0	-	+
B6	5	-	-
C5	29	-	+

C6	18	-	-
D5	39	-	-
D6	0	-	+
E5	46	+	+
E6	23	+	+

At the isolation stage, in addition to the emergence of Lactic Acid Bacteria (LAB) isolates, fungal growth also occurred on the surface of MRSA and CaCO<sub>3</sub> media. The growth of the fungus is supported by several factors such as temperature and saline environment<sup>18</sup>. This is in accordance with the environmental conditions of the sample, namely Mandai which was soaked with a high salt content and added with a bacterial dilution in the sample which also used 0.9% NaCl which caused a saline environment and became a natural habitat for halophilic and halotolerant fungi.

Based on the number of colonies obtained, it can be seen that the longer the Mandai fermentation time, the more the number of bacterial colonies that will grow. From the isolation results, only 1 sample produced a clear zone.



**Figure 1** Total of bacterial isolate colonies

In figure 1, it is known that in sample B5, which means sample B in a 10<sup>-5</sup> dilution, and in D6, which means sample D in a 10<sup>-6</sup> dilution, there are no bacterial isolates. This was due to the growth of fungi that filled the petri dish so that no bacterial isolates were found in the sample. there are bacterial isolates. This was due to the growth of fungi that filled the petri dish so no bacterial isolates were found in the sample. This happened for several reasons, one of which was that the bacteria were not colonies of Lactic Acid Bacteria (LAB) because isolation was not carried out using an anaerobic incubator.

### 3.1. Morphological Identification

In the macroscopic identification process, the things observed were shape (circular, irregular, spindle, filamentous, rhizoid), edges (entire, lobate, undulate, serrate filamentous, curled), elevation (flat, raised, convex, umbonate), and color.

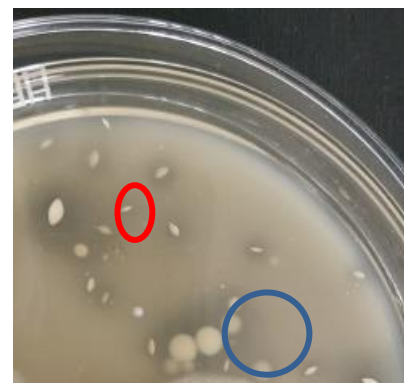
cream in color. This is in accordance with a study by Wardinal et al (2019) who isolated *Lactobacillus sp* in Sumatran orangutans which produced isolates of Lactic Acid Bacteria (LAB) with circular shape, entire edge, raised elevation and cream color.

**Table 3. Identification of bacterial isolate morphology**

Code	Morphology			
	Form	Edge	Elevation	Color
A5	Spindle	Entire	Raised	cream
A6	Circular	Entire	Raised	cream
B5	-	-	-	-
B6	Spindle	Entire	Raised	Cre m
C5	Spindle Circular	Entire	Raised	Cre m
C6	Spindle Circular	Entire	Raised, Umbonate	Cre m
D5	Circular	Entire	Raised	Cre m
D6	-	-	-	-
E5	Spindle Circular	Entire	Raised	Cre m
E6	Spindle Circular	Entire	Raised	Cre m

Note: (-) = No bacterial isolates

From the results of the macroscopic identification, it was found that the shape of the bacterial colonies was spindle and circular with entire edges, the elevation was dominated by raised shapes and



**Figure 2 Morphological form of bacterial isolates (red : spindle shape, blue: circular shape).**

Meanwhile, isolates from Lactic Acid Bacteria (LAB) of the genera *Lactobacillus sp* and *Bifidobacterium sp* were also found in spindle form<sup>18,19</sup>. Therefore, the results of macroscopic identification can confirm that the bacteria obtained are included in the Lactic Acid Bacteria (LAB).

### Gram Staining

The results obtained, it is known that there are bacterial isolates that are included in gram-positive bacteria

which are marked by the persistence of the purple color on the final result of gram-bacterial staining and gram-negative bacteria which are marked by the fading of the purple color to red after dripping with gram D dye (safranin).

**Table 4. Identification of bacterial isolate morphology**

No	Code	Gram	Shape
1	A5	+	Basil
2	A6	+	Basil
3	B5	x	x
4	B6	+	Basil
5	C5	+	Basil
6	C6	+	Basil
7	D5	+	Basil
8	D6	x	X
9	E5	+	Basil
10	E6	+	Basil

Note:

(x) = No bacterial isolates

(+) = Gram-Positive Bacteria

(-) = Gram-Negative Bacteria

From microscopic identification carried out by gram staining on the results of bacterial isolates, it was found that bacterial isolates showed a purple color and were in the form of bacilli in all isolates. These results are following the statement from Agustine et al (2018)<sup>20</sup> which states that gram-positive bacteria with a bacillus shape are one of the characteristics of Lactic Acid

Bacteria (LAB) which are included in the *Lactobacillus genus*.

### Biochemical Test

The catalase test is a biochemical test that aims to determine the physiological properties of bacteria in the ability to produce catalase enzymes which are characterized by the formation of gas bubbles where the formation of Oxygen (O<sub>2</sub>) gas comes from the breakdown of Hydrogen Peroxide (H<sub>2</sub>O<sub>2</sub>) by the bacterial catalase enzyme<sup>21</sup>. Based on the results obtained, only isolates E5 and E6 showed catalase-negative results. Lactic Acid Bacteria (LAB) are not bacteria that can produce catalase enzymes that convert Hydrogen Peroxide (H<sub>2</sub>O<sub>2</sub>) into water (H<sub>2</sub>O) and oxygen (O<sub>2</sub>) because LAB is a facultative anaerobic bacterium that predominantly lives in an environment without oxygen (O<sub>2</sub>)<sup>22</sup>. The other isolates showed the presence of air bubbles which meant the bacteria formed oxygen gas (O<sub>2</sub>).

**Table 4. Bacterial isolate biochemical test**

No	Code	Catalase Test	TSIA Test				
			Glu	Lac	Suc	Ga s	H <sub>2</sub> S
1	A5	+	-	-	-	-	-
2	A6	+	-	-	-	-	-
3	B5	x	x	x	x	x	x
4	B6	+	+	+	+	+	-
5	C5	+	+	+	+	+	-
6	C6	+	+	+	+	+	-
7	D5	+	-	-	-	-	-
8	D6	x	x	x	x	x	x
9	E5	-	+	+	+	-	-
10	E6	-	+	+	+	-	-

Note:

(x) = No identified bacterial isolates

(+) = Gram-Positive Bacteria

(-) = Gram-Negative Bacteria

Triple Sugar Iron Agar (TSIA) test which can confirm the ability of bacteria to ferment lactose, sucrose and glucose as well as the ability of bacteria to form acid, gas and H<sub>2</sub>S<sup>12</sup>. The TSIA test will show positive results in the ability to ferment glucose, lactose and sucrose and produce acid if a yellow color forms on the surface and medium, while positive results in the ability to ferment glucose only if a red or pink color is formed on the surface of the medium and a yellow color on the bottom. medium and if the media is cracked and lifted, it proves that there is an ability to produce gas as well as the formation of H<sub>2</sub>S gas if a black ring appears on the bottom of the

media<sup>12,14</sup>. Based on the results obtained, only isolates B6, C5, C6, E5, and E6 showed that the isolates were able to ferment glucose, lactose and sucrose. This is another characteristic of the type of Lactic Acid Bacteria (LAB). According to Utama, et al (2018)<sup>21</sup>, Lactic Acid Bacteria (LAB) genus *Lactobacillus* is able to hydrolyze cellulose into simple oligosaccharides which means that LAB has the ability to produce glucose, lactose and sucrose. The ability of Lactic Acid Bacteria to hydrolyze this can be useful for improving digestion and breaking down dietary fiber in the digestive tract.

## CONCLUSION

Mandai which is fermented using a salt solution in a closed state produces Lactic Acid Bacteria (LAB). The difference in Mandai fermentation time caused variations in bacterial isolates, namely the longer the fermentation was carried out, the more bacterial isolates were obtained. The examination of bacteria from Mandai showed positive results that bacteria could ferment carbohydrates in sample codes B6, C5,

C6, E5, and E6 as evidenced by the TSIA test and negative catalase results in sample codes E5 and E6 as evidenced by the Catalase test so that it can be said that only bacterial isolates from samples of Lactic Acid Bacteria (LAB) were obtained because they were able to ferment carbohydrates and did not have catalase enzyme.

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