

Characteristics of lactic acid bacteria isolated from palm sugar from West Sumatra, Indonesia and their potential as a probiotic

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Manuscript received: 21 March 2021. Revision accepted: 17 April 2021.

Abstract. Ramadhanti N, Melia S, Hellyward J, Purwati E. 2021. Characteristics of lactic acid bacteria isolated from palm sugar from West Sumatra, Indonesia and their potential as a probiotic. *Biodiversitas* 22: 2610-2616. Palm sugar is one of the sugar products from palm sap processing. It is made by reducing the water content in the sap to certain water content and is used as a natural sweetener. This study used palm sugar from 3 producers, i.e. Marapalam Gula Semut (MGS), Tetesan Gula Semut (TGS) and Sungayang Gula Semut (SGS) in Sungayang, West Sumatra, Indonesia. This study aimed to isolate and identify lactic acid bacteria (LAB) from palm sugar, potentially as a probiotic, using the 16S rRNA method. The probiotic potential of bacteria was selected based on morphology, biochemical properties, resistance to acidic pH 3 and 0.3% bile salts, antimicrobial activity against pathogenic bacteria. The results showed that lactic acid bacteria isolated from palm sugar are Gram-positive rod-shaped (Basil), negative catalase, and homofermentative. The total colonies of lactic acid bacteria ranged from 50×10^7 cfu/g - 117×10^7 cfu/g. The MGS lactic acid bacteria had 74.71% viability resistance in acidic pH 3, 65.52% viability against bile salts (0.3% ox gall). The diameter of inhibition against *Escherichia coli* O157 was 18.20 mm, *Propionibacterium acnes* (18.00 mm), *Acinetobacter baumannii* (14.19 mm), and *Listeria monocytogenes* (16.35 mm). The phylogenetic tree based on 16S rRNA showed that the MGS isolate has a similarity of 99.92% to *Lactobacillus fermentum* strain 1743. It can be concluded that *Lactobacillus fermentum* strain 1743 isolated from palm sugar has potential as a probiotic.

Keywords: 16S rRNA, lactic acid bacteria, *Lactobacillus fermentum* strain 1743, palm sugar, probiotic

INTRODUCTION

Sugar palm (*Arenga pinnata*) is a plant whose parts can be used for human needs. Each palm sugar has a different sap composition containing water, sucrose, reducing sugar, other organic materials, and inorganic materials. Palm sugar contains high glucose and sucrose compared to sugar cane. Sap raw materials are cooked until brown and thickened, followed by cooling and crystallization. Crystallization is performed by stirring using a wooden spoon to form sugar crystals. After that, it is sifted continuously to obtain a uniform grain size until the granules pass through the sieve, and then, the sugar is packed.

BAL can use sucrose contained in palm sugar in order to support human health. According to Joseph and Layuk (2012), palm sugar has a high carbohydrate content, i.e., sucrose ranging from 75-86%. Carbohydrate content in palm sugar is in the form of simple sugars, namely fructose and disaccharides. This sugar that undergoes glycolysis in aerobic condition produces pyruvate, but, glycolysis under anaerobic conditions produce lactic acid. The formation of lactic acid is caused by the lactase enzyme produced by lactic acid bacteria. Lactic acid bacteria are Gram-positive cocci, catalase-negative, and non-sporulating bacteria, and they produce lactic acid by fermenting glucose (Romadhon et al. 2012). Lactic acid bacteria are paired, or chain-shaped, immobile, facultatively anaerobic, and mesophyll

and are divided into several genera (Syukur and Purwati 2013). Lactic acid bacteria are important groups of probiotic bacteria, including *Lactobacillus*, *Lactococcus*, *Streptococcus*, *Pediococcus*, *Carnobacterium*, *Enterococcus*, *Propionibacterium*, and *Leuconostoc* (Kocabay and Cetinkaya 2020). *Lactobacillus* is the largest genus of lactic acid bacteria (LAB) with 183 species. It is a Gram-positive bacteria shaped like rods that can ferment glucose and produce lactic acid (Zhang et al. 2016). Many studies have been carried out to identify and characterize lactic acid bacteria isolated from biological natural resources. *Lactobacillus delbrueckii* KH3 was isolated from palm sap (Manel et al. 2011), *Lactobacillus fermentum* L23 from buffalo milk (Melia et al. 2018), *Lactobacillus plantarum* Dad-13 from curd (Purwandhani et al. 2018) as well as *Lactobacillus pentosus* from okara (Aritonang et al. 2017).

Lactic acid bacteria can be used as a source of probiotics. According to FAO (2002), probiotics are living organisms that can provide health benefits and safe to be consumed as food supplements. Probiotic bacteria, which are widely known, are included in the lactic acid bacteria group and are safe microorganisms called food-grade microorganisms (Syukur and Purwati 2013). Lactic acid bacteria must have probiotic characteristics. Probiotics characteristics include antimicrobial activity, resistance to the digestive system such as gastric acid, bile, and pancreatic juices, having anticarcinogenic activity, colonizing the digestive tract, and being able to increase the

ability of intestinal absorption (Syukur et al. 2011). Mojgani et al. (2015) stated that in general, probiotic bacteria must have essential properties such as resistance to the stomach and intestinal conditions, where acid and bile are factors affecting growth and survival. Isolation and identification of lactic acid bacteria from palm sugar are essential to be carried out to obtain new species of lactic acid bacteria from palm sugar. Also, the utilization of regional biological resource products will introduce regional specialties, especially West Sumatra.

MATERIALS AND METHODS

Sampling

This study used palm sugar from three different producers namely Marapalam Gula Semut (MGS), Tetesan Gula Semut (TGS) and Sungayang Gula Semut (SGS) in Nagari Andaleh Baruh Bukik, Sungayang Sub-District, Tanah Datar Regency, West Sumatra, Indonesia (Figure 1).

Palm Sugar Production

The raw material of palm sugar is sugar palm sap. The sap is provided and cooked for 8-10 hours at 100°C until brown and thickened, followed by cooling at 40°C for 1 hour and crystallization. Crystallization was performed by stirring using a wooden spoon to form sugar crystals. After that, it was sifted with an 18 mesh sieve to uniform the grain size. Sugar granules that did not pass must be refined, and then, it was performed continuously until the granules passed through the sieve. Finally, the sugar was packed (Figure 2).

Isolation and identification of Lactic Acid Bacteria

Palm sugar was weighed as much as 1 gram, then put in a test tube containing 9 ml of the MERCK de-Mann Rogosa Sharpe (MRS) broth solution, then it was vortexed until homogeneous. The result was called a 10^{-1} dilution, then was incubated for 24 hours in an incubator at 37°C.

100 μ L of the dilution was put into the Eppendorf tube containing 900 μ L of the MERCK de-Mann Rogosa Sharpe (MRS) broth solution. Furthermore, serial dilution was carried out up to 10^{-8} . From a 10^{-8} dilution, 100 μ L of the sample was cultivated by the spread method on a Petri dish containing MRS Agar (MERCK), then it was flattened with a stick. The inoculum was stored in an anaerobic jar then incubated in an incubator for 48 hours at 37°C. After 48 hours, a single colony of lactic acid bacteria was characterized. The isolate with yellowish-white slippery round was transferred to MRS Agar (MERCK) medium for colony purification using the streak method with ose needle, then incubated for 24 hours at 37°C (Shafakatullah and Chandra 2014).

Biochemical properties test

The Fermentative Type Test was carried out by inserting lactic acid bacteria isolates in 5 ml of MERCK MRS Broth. Then, the Durham tube was inserted upside down. It was incubated for 48 hours at 37°C. The presence of air bubbles in the Durham tube was observed (Rashid et al. 2014).

The catalase test was carried out by taking out lactic acid bacteria using a loop needle and then scratched on the object-glass. It was dripped with 3% hydrogen peroxide (H_2O_2) using a 50 μ L pipette, and gas presence was observed (Public Health England 2014).

Acid resistance test

Five hundred μ l of enriched LAB culture was inserted into a test tube containing 4.5 ml MRS Broth and then added with several drops of HCL to get pH three as the pH of stomach acid. The solution was diluted to a 10^{-6} dilution, then 100 μ l of dilution was cultivated on a petri dish containing MRS agar medium. It was incubated for 48 hours. After incubation, the number of colonies and their viability were calculated (Sunaryanto and Marwoto 2013).

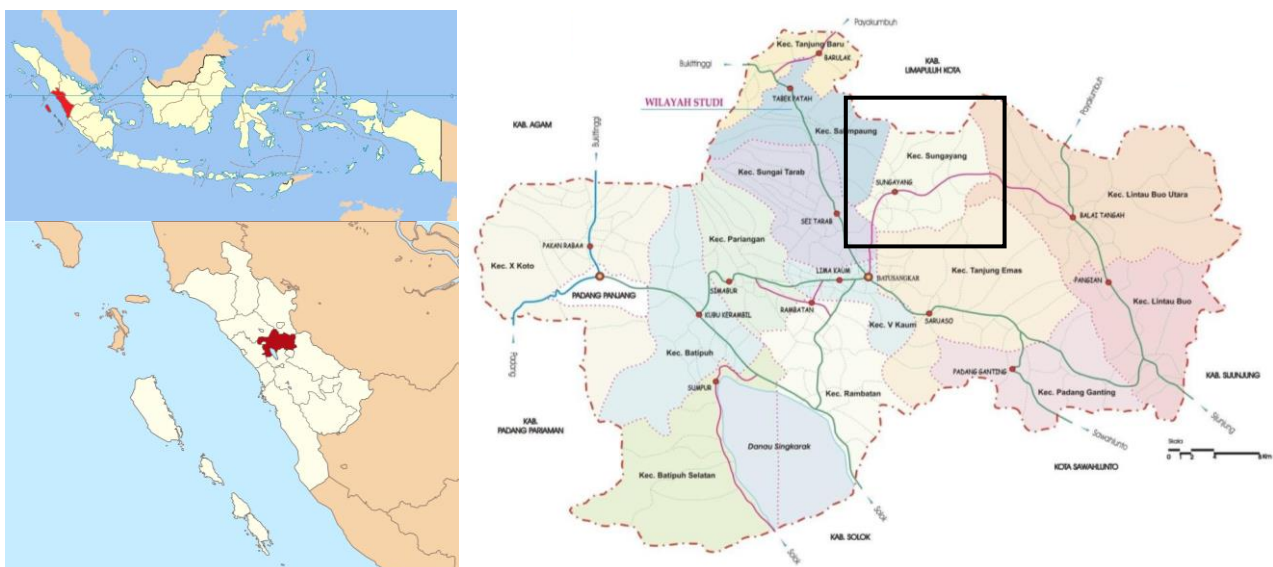


Figure 1. Map of Sungayang Sub-district (box), Tanah Datar District, West Sumatra Province, Indonesia (3° - 30° S, 98° - 101° E)



Figure 2. Palm sugar processing: A. Cooked sap, B. Stirring until brown and thickened, C. Sieved and filtered, D. Packed palm sugar

Bile salt resistance test

One ml of bacterial culture was inoculated in 9 ml of MRS Broth and incubated at 37°C for 24 hours. Furthermore, 1 ml of bacterial culture from MRS Broth was inserted into a test tube containing 9 ml of MRS Broth solution without ox gall (control) and in MRS Broth with 0.3% ox gall for 24 hours. Cultures with and without 0.3% ox gall (control) were diluted to 10^{-6} and planted by spread method onto MRS agar medium and incubated at 37°C for 48 hours. The number of viable bacteria was calculated as Colony Forming Units (CFU). After that, the LAB viability was calculated (Sunaryanto and Marwoto 2013).

Antimicrobial and antibiotic activity

The well-diffusion test carried out the antimicrobial activity test. Cell-free supernatant was obtained from lactic acid bacteria grown in MRS Broth (MERCK) for 24 hours at 37 °C under anaerobic conditions and centrifuged at 10,000 rpm for 5 minutes at 4°C. Fifty μ L of the supernatant was inserted into the well (6 mm), punched with a cork borer in Nutrient Agar. Nutrient agar has been inoculated previously with 0.2% of pathogenic bacteria. As a control, it was compared with antibiotics (penicillin 10 μ g, kanamycin 30 μ g, ampicillin 10 μ g). The clear zone was observed after 24 hours of incubation (Yang et al. 2012).

Molecular Identification of Lactic Acid Bacteria using 16S rRNA

Lactic acid bacteria were cultured in MRS broth at 37°C for 24 hours. Genomic DNA isolation was carried out using Extrap Soil DNA Kit Plus Ver.2. The polymerase chain reaction (PCR) amplification of 16S rRNA isolates used 16S rRNA gene fragments of ~ 1.5 KB as universal primers. Initial denaturation was carried out at 95°C for 5 minutes with 25 cycles, followed by denaturation at 94°C for 1 minute. Annealing was carried out at 56°C for 1 minute, extension at 72°C for 1.5 minutes, and a final extension at 72°C for 7 minutes. The resulting DNA was separated using electrophoresis at 100 V for 21 minutes using 1% agarose in \times 1 TAE buffer. A gel washer documentation system was used to generate an image of the tape on the gel. Purification was performed using a rapid gene gel/PCR extraction kit (Nippon Genetics, Germany). The sequences were analyzed using the BLAST program in the NCBI gene bank database available at <http://blast.ncbi.nlm.nih.gov/Blast.cgi>. The sequence

alignment was made using the Bioedit application, and the phylogenetic tree was created using the MEGA 6 application.

RESULTS AND DISCUSSION

Morphological Identification of Lactic Acid Bacteria

The density of lactic acid bacteria in MGS palm sugar was 117×10^7 CFU/g, TGS palm sugar was 76×10^7 CFU/g, and SGS palm sugar was 50×10^7 CFU/g. According to the FAO criteria (2002), the colony yield of lactic acid bacteria should be 10^6 - 10^8 CFU/gram. Lactic acid bacteria isolated from palm sugar were yellowish-white with circular colony shapes and raised elevations (Table 1); however, Juliarysi et al. (2018) found that lactic acid bacteria's characters were circular, creamy white, and raised in MRS agar.

Furthermore, the lactic acid bacteria were observed microscopically for Gram stain. Gram stain determines bacteria as Gram-positive or Gram-negative. It is indicated by the ability of bacteria to absorb color reagent. The Gram staining results showed that bacteria isolated from MGS, TGS, and SGS palm sugars were rod-shaped and Gram-positive. Bacteria treated with crystal violet that remain purple although washed with alcohol and exposed to red safranin are Gram-positive bacteria, while Gram-negative bacteria will turn red (Unus 2005). Lactic acid bacteria have the characteristics of Gram-positive, rod-shaped or round, facultatively anaerobic, nonsporulating, and produce lactic acid bacteria as the main product of carbohydrate fermentation (glucose, fructose, and sucrose) (Salminen et al. 2004).

Biochemical properties

The biochemical test results showed that bacteria isolated from palm sugar are catalase-negative and homofermentative (Table 2). A negative catalase reaction was indicated by the absence of bubbles, which means that there is no formation of oxygen gas due to H_2O_2 breakdown by the catalase enzyme produced by bacteria. A previous study by Yelnetty et al. (2014) showed that there are 26 isolates from goat milk with spontaneous fermentation, and there is a negative catalase reaction. Homofermentative was indicated by the absence of bubbles in the Durham tube. It is in line with a study by Melia et al. (2019) that the lactic acid bacteria *Pediococcus acidilactici* PB22 strain is a homofermentative isolate.

Resistance of Lactic Acid Bacteria to acidic conditions

The viability of lactic acid bacteria to acidic conditions (pH 3) was in the range of 48.39 - 74.71% (Table 3). Each isolate has different viability because each isolate has a different ability to survive at gastric pH (acid). MGS had the highest viability of 74.71%. Lactic acid bacteria, which have the potential as probiotic, must be resistant to gastric pH, ranging from 2-3, caused by the secretion of gastric juices. The viability of lactic acid bacteria isolated from milk and cow dung at pH 3 ranged from 61.44-81.25% (Jain et al. 2017). Melia et al. (2018) showed that the lactic acid bacteria isolate *Lactobacillus fermentum* strain L23 can survive at pH 2 with viability of 55.64-73.94%. Lactic acid bacteria from palm sugar have higher viability than *Lactobacillus rhamnosus* from buffalo milk from Karnataka, India, with 30% viability at pH 3 for 3 hours (Shafakatullah and Chandra 2014). The population density of lactic acid bacteria with potential probiotics must be $> 10^6$ CFU/gram after incubation at pH 3.0 (Azat et al. 2016). It indicates the survival rate of lactic acid bacteria in the pH of the digestive tract. Harun et al. (2020) stated that the higher the lactic acid bacteria's viability, the higher the resistance of bacteria or isolates of lactic acid bacteria to gastric pH.

The resistance of Lactic Acid Bacteria to bile salt

Lactic acid bacteria from palm sugar was tested against 0.3% bile salts for 4 hours. The result showed that the viability ranged from 30.43 to 65.52%, with the highest viability was MGS isolate (Table 4). This study indicated that the LAB isolate from palm sugar could live at a concentration of bile salts of 0.3-0.5% as in the human body. The viability of LAB from palm sugar in 0.3% bile salts after 4 hours of incubation was varied (Table 4). MGS isolate had a viability of 65.52% under the bile salts conditions. It showed that MGS could survive better than TGS and SGS. The higher the bacterial viability obtained, the higher the resistance of probiotic bacteria to bile salts. An effective probiotic must be able to withstand the conditions of bile salts secreted by the liver. Bile salts reduce the number of bacteria by destroying their cell walls. MGS isolates had higher viability than *Lactobacillus fermentum* strain IMAU70167 isolated from buffalo milk with 32.23-56.13% viability in 0.3% bile salts for 4 hours (Melia et al. 2018). Anandharaj and Sivasankari (2014) found that *Lactobacillus oris* strain HM168 isolated from breast milk had a viability of 20.1-26.9% at 0.3% ox gall for 3 hours. The lactic acid bacteria strains isolated from buffalo milk in Karnataka, India can survive at bile salts concentrations of 0.3% and 0.5% bile salts but did not survive at 1% (Shafakatullah and Chandra 2014). According to Harun et al. (2020), lactic acid bacteria's resistance to bile salts is related to the enzyme Bile Salt Hydrolase (BSH), which hydrolyze conjugated bile salts, thereby reducing the toxic effect on cells.

Antimicrobial activity of Lactic Acid Bacteria

The highest resistance to acids and bile salts of MGS lactic acid bacteria was selected for antimicrobial activity against pathogenic bacteria. MGS lactic acid bacteria had a

clear zone against *E. coli* 0157 (18.20 mm), *Propionibacterium acnes* (18.00 mm), *Acinetobacter baumannii* (14.19 mm), and *Listeria monocytogenes* (16.35 mm) (Table 5).

Escherichia coli O157 was the most sensitive to MGS isolate with a clear zone diameter of 18.20 mm. The diameter of MGS inhibition against *E. coli* O157 was higher than the inhibition of the lactic acid bacteria isolated from tempoyak (12 mm) (Juliarsi et al. 2018). However, the MGS inhibition diameter against *Listeria monocytogenes* was relatively lower than *Lactobacillus fermentum* strain L-23 isolated from Agam buffalo milk (19 mm) (Melia et al. 2017). MGS inhibition was higher than *Pediococcus acidilactici* strain PB22 isolate from Bekasam (5.10 mm) (Melia et al. 2019) and has been applied in the manufacture of yogurt probiotic (Melia et al. 2020). The inhibition zone of MGS against *Acinetobacter baumannii* was relatively low (14.19 mm), but it was higher than *Lactobacillus rhamnosus* GG (11.76 mm) (Dallal et al. 2017).

Pan et al. (2009) categorized the antibacterial activity based on the diameter of the clear zone as follows: low antimicrobial activity (0-3 mm), moderate antimicrobial activity (> 3-6 mm), and high antimicrobial activity (> 6 mm). Some lactic acid bacteria had antimicrobial activity against *L. monocytogenes* and also have probiotic potential. Organic acids and hydrogen peroxide produced by lactobacilli can inhibit the growth of Gram-positive and Gram-negative bacteria, while bacteriocin has more effect on Gram-positive bacteria (Vasiee et al. 2014). Lactic acid bacteria produce lactic acid, the main glucose fermentation product (Arena et al. 2017).

Table 1. Macroscopic identification of lactic acid bacteria isolates

LAB isolate	Color	Colony form	Margin	Elevation
MGS	Yellowish-white	Circular	Entire	Raised
TGS	Yellowish-white	Circular	Entire	Raised
SGS	Yellowish-white	Circular	Entire	Raised

Notes: MGS (Marapalam Gula Semut), TGS (Tetasan Gula Semut), SGS (Sungayang Gula Semut)

Table 2. Catalase test and fermentation type of lactic acid bacteria isolates from palm sugar

LAB isolate	Catalase test	Fermentation type
MGS	Negative (-)	Homofermentative
TGS	Negative (-)	Homofermentative
SGS	Negative (-)	Homofermentative

Table 3. The viability of Lactic Acid Bacteria from palm sugar at acidic conditions

LAB isolate	Number of colonies (CFU/gram)		Viability (%)
	pH control	pH 3	
MGS	87 x10 ⁷	65 x10 ⁷	74.71
TGS	68 x10 ⁷	40 x10 ⁷	58.82
SGS	62 x10 ⁷	30 x10 ⁷	48.39

Table 4. The viability of Lactic Acid Bacteria isolated from sugar palm to bile salts

LAB isolate	Number of colonies (CFU/gram)		Viability (%)
	Control	0.3% Oxgall	
MGS	87 x10 ⁷	57 x10 ⁷	65,52
TGS	68 x10 ⁷	37 x10 ⁷	54,41
SGS	62 x10 ⁷	25 x10 ⁷	30,43

Table 5. The clear zone diameter of MGS lactic acid bacteria against 4 pathogenic bacteria

Inhibition source	The diameter of the clear zone (mm)			
	<i>E. coli</i> 0157	<i>P. acnes</i>	<i>A. baumannii</i>	<i>L. monocytogenes</i>
MGS	18.20	18.00	14.19	16.35
Ampicillin	-	-	5.05	-
Kanamycin	14.95	16.80	15.20	14.10

Results of 16S rRNA amplification using PCR

Amplification of the 16S rRNA gene isolate of MGS lactic acid bacteria from palm sugar was presented in Figure 3. PCR fragment size of 1542 bp used R Primer (16S-1492R, Tm 47 ° C, 5'-GTT TAC CTT GTT ACT ACT-3') and F (16S-27F, Tm 54,3 ° C, 5'-AGA GTT TGA TGCC CTC AG-3').

The phylogenetic tree based on the 16S rRNA gene sequence can be seen in Figure 4. The sequencing of palm sugar isolates was compared with Gen Bank data using the BLAST program on the NCBI website (<http://www.ncbi.nlm.nih.gov>), showing a similarity level of 99.93% with the *Lactobacillus fermentum* strain 1743. Therefore, it can be concluded that the MGS lactic acid

bacteria isolated from the palm sugar is *L. fermentum* strain 1743. This lactic acid bacteria isolate a new strain found in palm sugar.

In conclusion, the lactic acid bacteria isolated from palm sugar had a total colony of 50 x 10⁷ CFU/g - 117 x 10⁷ CFU/g in rod-shaped, Gram-positive, catalase-negative, and homofermentative. The MGS lactic acid bacteria isolated from palm sugar in Marapalam Sugar Ant (MGS) had the viability of 74.1% at pH 3, 65.52% in 0.3% bile salts, and the highest antimicrobial activity against *E. coli* O157 with an inhibition zone of 18.20 mm. The phylogenetic tree results based on 16S rRNA, MGS isolates from the producer Marapalam Sugar Ants in Sungayang Tanah Datar District were identified *Lactobacillus fermentum* strain 1743.

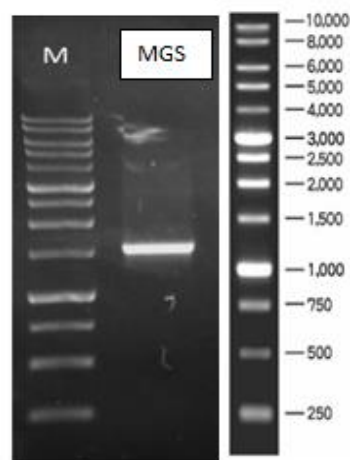


Figure 3. PCR amplification of ribosomal RNA using 11492R and 27F. MGS is a lactic acid bacteria isolated from palm sugar

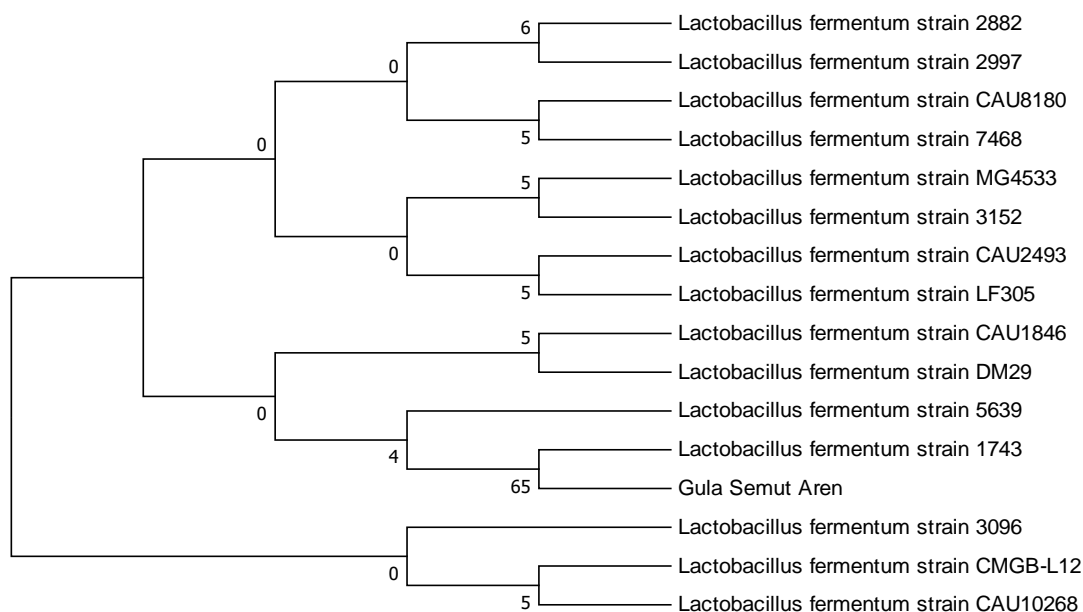


Figure 4. Phylogenetic Tree of *Lactobacillus fermentum* strain 1743

ACKNOWLEDGEMENTS

This study was supported by the Laboratory of Livestock Products Technology, Faculty of Animal Husbandry, Andalas University, Padang, Indonesia. The author would like to thank the Institute for Community Service Research (LPPM) Andalas University through the excellent basic research scheme for professor publication research clusters with contract number T/15/UN.16.17/PT.01.03/KO-PDU-KRPIGB/2020, to Prof. Endang Purwati.

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