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Isolation and Identification of Lactic Acid Bacteria in Sinjai Typical Drinks (MINAS)

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ABSTRACT

A fermented drink produced locally in the Sinjai area that is well known in the local community as well as widely is Minas. Minas, which means a typical Sinjai drink, used to be known as Irex. However, over time this drink was replaced with the name Minas because it was only produced in Sinjai City and used as a typical Sinjai drink has properties and benefits to increase body stamina due to fatigue after doing daily activities or work. Minas are made from cassava tapai, young coconut, eggs, coconut water, sugar water, honey, and milk and can be added with other fruits such as durian. Curved tape is a type of food produced from the fermentation process of substrates or carbohydrate foodstuffs using yeast yeast which belongs to the group of lactic acid bacteria. To determine the type of lactic acid bacteria that play a role in the fermentation process of minas drinks by isolating and identifying morphology using the gram staining method, biochemical tests using catalase tests, gas production and carbohydrate fermentation tests. Furthermore, molecular identification uses 16S RNA. The DNA sequencing results were analyzed with MEGA 6 software with the BLASTn algorithm at NCBI. Based on the results of the identification of lactic acid bacteria, homologous nucleotides were detected with the bacteria *Bacillus subtilis, Bacillus* and *Streptococcus sp* which are a group of lactic acid bacteria that are able to ferment carbohydrates to produce lactic acid.

Keywords:
isolation, identification, lactic acid, bacteria, fermented, drink, Sinjai

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INTRODUCTION

Sinjai is one of the districts in South Sulawesi Province that has a very good geographical area and conditions and has a variety of diversity of life ranging from aspects of culture, language, religion and even a diversity of regional food and beverages. One of them is the household industry, the prominent household industry in the Sinjai area is the fermented beverage industry. This drink is known as Minas which is a typical drink of the Sinjai region (Rahmah *et al.,* 2021).

Minas is a typical Sinjai drink that has properties and benefits to increase body stamina due to fatigue after doing daily activities or work. Minas is made from cassava tapai, eggs, young coconut, coconut water, sugar water, honey, and milk and can be added with other fruits such as durian. Minas is a fermented drink made from cassava tape with the addition of several other ingredients so that it cannot last long. The shelf life of minas is affected by the storage temperature, which is a week if stored in the freezer and 3-5 days if only in the refrigerator (Rahmawati, 2019). Minas drinks are produced from the fermentation process of substrates or carbohydrate foods using yeast which belongs to the lactic acid bacteria group (Sulistiani and Hidayat, 2020).

Lactic acid bacteria (BAL) are bacteria that can convert carbohydrates into lactic acid which is safe if added to food because it does not produce toxins and produce bacteriocins. The existence of BAL has various potentials in lowering cholesterol, namely bacteria *Lactobacillus fermentum* and *Lactobacillus acidophilus bacteria* (Fadhilah *et al*., 2015). Research. previously showed the results of isolation and characterization of BAL in soybean immersion liquid waste, namely the genus *Lactobacillus* (Amaliah *et al*., 2018). Based on previous research, BAL isolate has the morphological characteristics of basil or rod, gram positive, catalase negative, does not produce gas, and slow acidification speed. The process of identifying bacteria in general by going through the process of isolation from the sample with a dilution technique to obtain pure isolates, then morphological identification by gram staining to determine the characteristics of the shape and type of gram-positive bacteria or gram-negative bacteria, while the biochemical activity test is catalase test, gas production test and similarity speed test to obtain chemical reaction characteristics (Nudyanto and Zubaidah, 2015).

MATERIALS AND METHODS

Time and Place

This research was conducted at the Laboratory of Microbiology and Molecular Biology of the Hasanuddin Medical Research Center (HUMRC). Samples were taken at the Minas beverage production site, Sinjai Regency, South Sulawesi. The PCR samples were sent to the sequencing company 1st Base Malaysia.

Materials and Tools

Isolate the DNA samples using *the PrestoTM g DNA* Bacteria Kit method and measure DNA using *the Nano Drop 2000 DNA spectrophotometer*, *Polymerase Chain Reaction* (PCR) machine. Master Mix 2 *(Promega, Madison, WI, USA),* Primers used 63f (5'CAGGCCTAACACATGCAAGTC-3') and 1387r (5'-GGGCGGWGTACAAGGC-3'). Samples of Minas drinks, *Mann Regosa Sharpe Agar* (MRSA) media, *Triple Sugar Iron Agar* (TSIA) media, *Nutrient Broth* (NB), cotton, Hydrogen Peroxide (H_2O_2) and aquades.

Sample suspension manufacturing.

A total of 1 ml of minas sample was put into a test tube containing 9 ml of sterile aquades and diluted from 10^{-1} to 10^{-7} then homogenized using vortex.

Isolation and purification of lactic acid bacteria

Addition of $CaCO_3$ 1 g to media *de Mann Regosa Sharpe Agar* (MRSA). 1 ml of dilution 10^{-4}, 10^{-5}, 10-6, and 10-7 was taken from the prepared sample suspension, dripped on MRSA media and

then flattened using a spider until dry. then incubated at a temperature of 37^{oC} for 2 x 24 hours. The colonies formed are purified using the quadrant scratching method, then the separate colonies are transferred to the oblique culture medium as stock.

Gram staining

The method is done (Bergey, 1984) in aseptic conditions, take 1 ose of bacterial culture, flatten it on the glass of the object and then fix it on the fire. Then drip with a solution of cristal violet and let it sit for 1 minute, then wash it with running water and dry. Next, it is dripped with iodine solution and let it sit for 1 minute, then wash it with running water and dry. Then wash with a bleach solution for 30 seconds, wash under running water and dry. After that, drip with safranin solution, let it sit for 2 minutes, then wash it with running water and dry. Then observe with a microscope with a magnification of 1000x.

Biochemical Tests

The catalase test was carried out by pure isolate placed on the glass of the object and then dripped with H_2O_2 to observe the presence or absence of gas bubbles produced. The gas production test was carried out by growing bacterial isolate in medical MRS broth on a test tube containing a durham tube for 2 x 24 hours at a temperature of 37^{o}C. Observations were made by looking at the presence of air bubbles formed in durham tubes. Isolates that produce bubbles are heterofermentative bacteria, which are able to produce CO_2 gas, while isolates that do not form bubbles are called homofermentative bacteria. Testing of glucose fermentation using *Triple Sugar Iron Agar* (TSIA) media in test tubes by piercing the isolate on the medium perpendicularly. Then the culture was incubated at 37ºC for 24 hours and a change in the color of the medium was observed. If the slant of the medium is red and the butt is yellow, then the bacteria are able to ferment glucose. If the slant and butt of the medium are yellow, then the bacteria are able to ferment glucose, lactose and sucrose.

Molecular Identification

Pure single bacteria were grown in 50 mL of *Nutrient Broth* (NB) and incubated at 37°C for 24 hours. The bacterial cells are then centrifuged at 9,500 g for 1 minute. The DNA of the selected bacterial genomes was extracted using *the PrestoTMg DNA* Mini Bacterial Kit (Genaid). The 16S rRNA gene was amplified using a *Polymerase Chain Reaction* (PCR) machine. A total of 50 ml of PCR Mix was made with the composition: 25 ml of GoTaq *Green Master Mix 2 (Promega, Madison, WI, USA),* 2.5 ml (100 pmol) for each primer: 63f (5'CAGGCCTAACACATGCAAGTC-3') and 1387r (5'-GGGCGGWGTACAAGGC-3'), 0.3 ml (64.4 ng/μL) of DNA template and 19.7 ml of nuclease-free water. Polymerase chain reaction (PCR) is performed under the following conditions: pre-denaturation at 94 °C for 5 min, denaturation at 92 °C for 30 sec, annealing at 58 °C for 30 sec, elongation at 72 °C for 1.5 min and final extension at 72 °C for 5 min with 35 cycles. Finally the temperature is lowered to 4 °C for 10 minutes to stop the PCR reaction. *Polymerase Chain Reaction* (PCR) products are purified and sequenced by sending them to a sequencing service company *(Genetica science).* The sequences were analyzed using the Bioedit program and then aligned with the 16S rRNA gene database using the BLAST-N program.

RESULTS AND DISCUSSION

Isolation and Purification of Lactic Acid Bacteria

In the results of isolation and purification of microbial isolates, microbial colonies obtained from 4 selected isolates showed the presence of a clear zone around the colony on MRSA medium when incubated for 2 × 24 hours at a temperature of 37°C. The clear zone is formed because $CaCO_3$ (Calcium carbonate) in the medium is alkaline so that the bacteria that form the clear zone have the ability to neutralize with acid production. (Khristnaviera and Meitiniarti, 2017). The selected isolate is suspected to be lactic acid bacteria with

an indicator of the formation of a clear zone around the colony and then purified using the square stroke method to produce pure isolate (Fig. 1).

Gram staining of lactic acid bacter isolate.

In the microscopic test, lactic acid bacterial isolate (BAL) was stained with grams to see the color and shape of the microbial isolate cells. The purple color indicates Gram-positive bacteria, while the red color indicates Gram-negative bacteria (Figure 2). The result of the gram staining to 4 isolates is a group of gram-positive bacteria marked by purple in bacterial cells, this purple color indicator is caused because the cell wall of Gram-positive bacteria contains more peptidoglycan layers, so that violet-iodine crystals that enter the bacterial cell cannot be washed by alcohol. As long as the purple crystal violet paint survives in the bacterial cell, the D gram paint (safranin) as a follow-up paint will no longer be absorbed, so the cell color will remain the same color as the first paint used (crystal violet paint). From the Gram staining, it can also be seen that the shape of isolated bacterial cells is in the form of paired rods (diplobacilli); (Pelczar and Leg, 2010); (Rini and Jamilatur 2020). Lactic acid bacteria are generally gram-positive bacteria (Pelczar and Chan, 2010) ; (Rini and Jamilatur, 2020).

Biochemical Tests

The results of the characterization of 4 isolates based on biochemical tests in Table 1. shows that the four isolates have the same properties, namely including gram-positive bacteria, catalase positive, able to ferment glucose and sucrose/lattose. However, the M22-Ee isolate is not capable of producing gas.

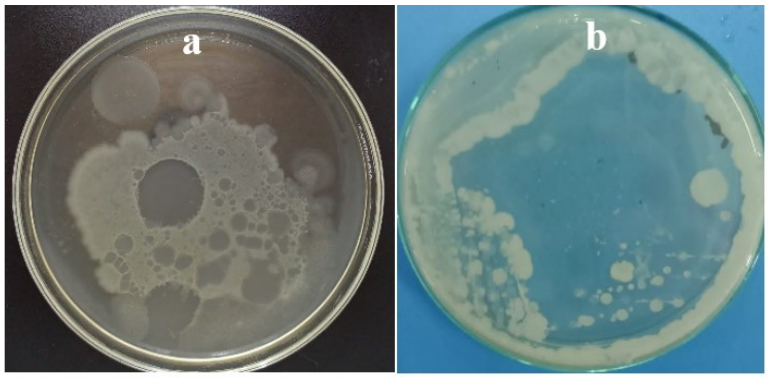

Figure 1. Isolation and purification of lactic acid bacteria
(a) Isolation at dilution 10^{-7} (b) purification of selected isolate quadrant scratching method

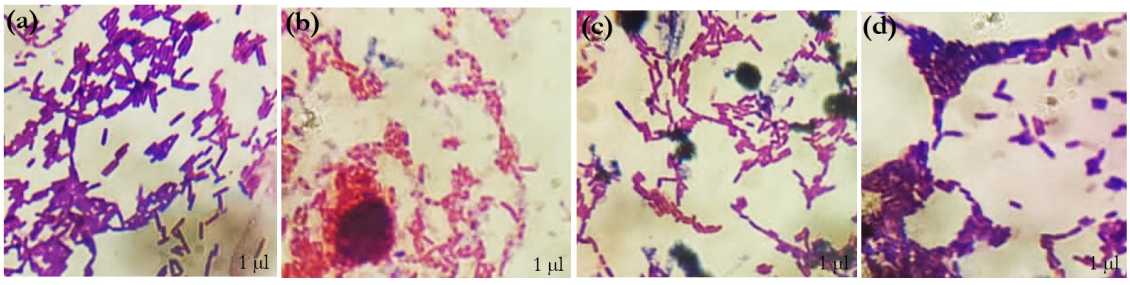

Figure 2. Gram staining of 4 lactic acid bacterial isolates
(a) M22-Aa (b) M22-Dd (c) M22-Ee and (d) M22-Gg at 1000x magnification

Catalase test with the formation of bubbles formed on the preparate after being dripped with H_2O_2, glucose fermentation test with color change in TSIA media whose slope part becomes red and yellow, then gas production test with bubbles formed on durham tubes. The results of the catalase test showed that 4 bacterial isolates were positive, characterized by the formation of bubbles in the preparations that had been dripped by H_2O_2. In contrast to the statement (Mergypta *et al*., 2014) that lactic acid bacteria are not able to produce the catalase enzyme used to break down hydrogen peroxide into dihydroxy oxide (H_2O) and oxygen (O_2).

Gas production test Isolates that produce bubbles are heterofermentative bacteria that are able to produce CO2 gas, while isolates that do not form bubbles are called homofermentative bacteria. The results of M22-Aa, M22-Dd and M22-Gg isolates (Amaliah *et al*., 2018) show that there are no bubbles formed in the durham tube so that the isolate does not produce gas so that the bacterial isolate is classified as homofermentative while the M22-Ee isolate has bubbles formed in the durham tube so that the isolate is able to produce gas, so it is classified as heterofermentative bacteria.

The results of the carbohydrate fermentation test showed a change in color on the surface of the media. Carbohydrate fermentationcan occur aerobically on the agar surface and anaerobically on the agar base. A positive test is indicated by a red color on the oblique part of the medium which indicates that bacteria ferment glucose, while if a yellow color is formed, then positive bacteria ferment lactose and sucrose. The results of the carbohydrate fermentation test showed that the isolates were positive in fermenting carbohydrates by forming a yellow color on the medium and showed that the isolates were positive for fermenting lactose and sucrose (Nudyanto & Zubaidah, 2015).

Based on Table 1. Showing simultaneous characteristics between M22-Aa, M22-Dd and M22-Gg isolates which are gram-positive groups of stem-shaped morphological forms, positive catalase test and positive carbohydrate fermentation and do not produce gas. However, some other studies have reported the results of the identification of lactic acid bacteria with negative catalase test characteristics; According to (Ismail & Yulvizar ., 2017); (Riadi *et al* ., 2017); (Detha , 2019) it also states that lactic acid bacteria are bacteria that produce acid in their carbohydrate metabolism, classified as Gram-positive, round or rod-shaped, catalase negative so that this study was carried out molecular tests to support the data obtained.

Molecular Identification

In this study, molecular examination was carried out by isolating bacterial DNA by PCR method using 16s RNA primer 63F/1387R and 16s bact 338/bact 1525 primer, the results showed the formation of DNA bands at a size of 1200bp (Fig. 3). The results of DNA isolate sequencing that has been analyzed with GeneBank data using the BLAST program from the nucleotide sequence data of the 16S rRNA gene succeeded in identifying 3 species of bacteria classified as lactic acid bacteria, namely *Bacillus subtilis*, *Bacillus sp* and *Streptococcus sp* with homology of 98-100% against strain type sequences in NCBI GenBank (Table 2).

Table 1. Morphological, physiological and biochemical characterization in MINAS beverages

No.	Inoculum	Colony Form	Grams	Catalase	Gas production	Glucose fermentation	Fermentation of sucrose/lactose
1	M22-AA	Trunk	+	+	-	+	+
2	M22-DD	Oval round	+	+	-	+	+
3	M22-EE	Trunk	+	+	+	+	+
4	M22-GG	Trunk	+	+	-	+	+

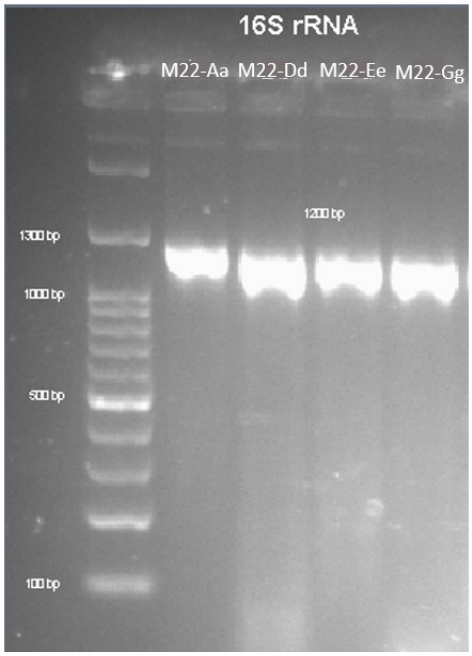

Figure 3. Results of bacterial isolate electrophoresis

Table 2. Results of BLAST Sequencing of Lactic Acid Bacteria

No.	Inoculum	Molecular identification	Similarity (%)	Accession Number
1	M22-AA	*Bacillus subtilis*	100	M58832.1
2	M22-DD	*Streptococcus sp*	98	CP013651.1
3	M22-EE	*Streptococcus sp*	99	MT482642.1
4	M22-GG	*Bacillus sp.*	99	MZ005634.1

Based on the results of molecular identification with sequencing analysis, the types of lactic acid bacteria found in minas drinks were *Bcacillus sp, Bacillus subtilis,* and *Streptococcus* groups. B. *subtilis* bacteria have motile properties and have various oxidation activities. Species *Bacillus sp.* determined based on physiological characterization characteristics with the results of the identification of *the species Bacillus sp* (Sahirman, 2021). Based on the characteristics, it is known that the bacteria has a positive catalase test, a positive starch hydrolysis test and its optimal growth temperature is 37ºC. A positive catalase test proves that *Bacillus sp* endophytic has a catalase enzyme (Puspita *et al.,* 2017).

In this study, M22-De and M22-Ee isolates based on the nucleotide arrangement of 16S rRNA were identified as belonging to the *Streptococcus bacterial group* (Table. 2), but in the biochemical test there were differences in the shape and results of the catalase test (Table. 1). Streptococcus *bacteria* are gram-positive (+), non-motile, non-endospore-forming, facultative anaerobic and homofermentative. Based on other studies, biochemical tests obtained negative results for cytochromes, catalase and oxidase, and positive for alphahemolytic with the characteristics obtained above these bacteria are classified as lactic acid bacteria (Brooks *et al.,* 2010); (Hasanah, 2014). The group of *Streptococcus bacteria* is an acid-producing bacterium that converts lactose into lactis, a positive chain spherical shape, without spores, can grow anaerobically. The bacteria are homofermented, the optimum temperature is 65 ^{0}C, the minimum pH is 4.3-4.8. The two isolates were identified as the same group, namely

Streptococcus sp. Based on the results of molecular identification, but the characteristics of biochemical tests are different, namely the ability to produce gas in small quantities so that it is possible to form gas or not at all. According to (Rompis *et al.*, 2018) *Streptococcus sp.* bacteria in carbohydrate fermentation tests, carbohydrates can be fermented but CO_2 gas is formed in very small amounts or not at all

CONCLUSION

The type of bacteria identified was Lactic Acid Bacteria (BAL) isolated in Sinjai (Minas) drinks in this study based on morphology, biochemical and molecular tests including the groups *Bacillus subtilis*, *Bacillus sp* and *Streptococcus sp.*

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