Isolation and identification lactic acid bacteria of honey-enriched functional beverage from cassava (*manihot esculenta*) tapai from Sinjai regency

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**Abstract.** Cassava (*Manihot esculenta*) tapai is a traditional food that is classified as a source of probiotics because it is known to contain lactic acid bacteria (LAB). Cassava tapai is processed by many people in Sinjai Regency to become a functional beverage and one of the ingredients added is honey which has a high sugar content as one of the sources of BAL nutrition. The purpose of this study was to isolate and identify the types of LAB with probiotic potential in functional drinks of cassava (*Manihot esculenta*) during freezing temperature storage. Functional drinks of cassava (*M. esculenta*) tapai were treated with the addition of honey namely M1 = 0% (control), M2 = 2% honey, M3 = 4% honey, M4 = 6% honey, M5 = 8% honey. Microbial isolation and characterization, probiotic testing, and molecular testing were carried out to identify the probiotic bacterial species in functional drinks of cassava. Based on the results of the study it can be concluded that the two isolates were identified as BAL with probiotic potential from the functional drinks of cassava (*M. esculenta*) namely *Pediococcus acidilactis* and *Weissella cibaria*.

1. Introduction

Sinjai is one of the districts of 24 regencies/cities in south Sulawesi province which borders three districts namely Bone Regency in the north, Bulukumba Regency in the south, and Gowa Regency in the west. Administratively, Sinjai does not experience the expansion of the district nor the village/Kelurahan. Sinjai remained divided into 9 sub-districts with 80 villages/Kelurahan [1]. The types of food crops that are cultivated in Sinjai District are rice, corn, cassava, sweet potato, and peanut. Cassava is one type of food that has a role as a complement of carbohydrate sources other than rice and corn. Cassava production in Sinjai County in 2017 amounted to 481 tons of wet yam [2].

Cassava contains four nutritional groups, namely carbohydrates, fats, proteins and minerals. The advantage of cassava is mainly on carbohydrates and fats which is the main source of fuel energy generation of the body. One of the processing of cassava is traditionally with fermentation process into cassava tapai. Fermentation in food processing is the conversion of carbohydrates into alcohol and carbon dioxide using yeast, bacteria, fungi or a combination of the three [3]. We found that a group of people in Sinjai district cultivate the cassava's tapai into a functional beverage known as Minas. At a glance, the functional drink is similar to an energy enhancer sold on the market, with a light yellow color with a slightly viscous texture. The main ingredient is cassava tapai with some other additives such as coconut water, milk, sugar and honey. In addition to the higher nutritional value of the original
ingredient, the tapai also contains lactic acid bacteria. Lactic acid bacteria is a bacteria producing large amounts of lactic acid as the end result of metabolism sugars (carbohydrates). The resulting lactic acid lowers the pH value of its growth environment and creates an acidic flavor. It also inhibits the growth of some other types of microorganisms. In addition, microorganisms also require a food supply that will be a source of energy and provide the basic chemical elements for cell growth [3].

Honey is one of the ingredients added in the preparation of the functional beverage formula that potentially lowers the pH and provides sugar as a nutrient for the growth of lactic acid bacteria. Some research suggests that both of these ingredients are cassava and honey tapai potentially producing lactic acid bacteria. Based on the background, this study aims to isolate and identify lactic acid bacteria found in the functional beverages based on cassava tapai.

2. Material and Method

2.1. Material

The tools that are in the form of HPLC (High Performance Liquid Chromatography) Shimadzu Prominence LC-20AD, LAF (Laminar Air Flow) Faster Bio60, autoclaved Tomy SX-500, Mamert oven, Inkubatar Mamert, incubator WTW TS 606/2-I, Waterbath Mamert, microscope and glass object, DNA thermal cycler (Applied Biosystems), electrophoresis + tip supply, UV lamp device + digital camera, viscometer DV-I Prime, moisture Analyzer DSH-50-1, vortex Thermolyne, pH meter, reaction tube, petri dish, erlenmeyer, chemical glass, mixing rod, measuring cup, pipette drops, ose, Bunsen, petri dish, measuring flask, analytical scales.

The material used is a functional beverage of cassava (M. esculenta) tapai, Indonesian organic honey obtained from the forestry faculty of Hasanuddin University, MRS Agar, MRS Broth, Aquadest, NaCl, cotton, sterile gauze, aluminium foil, dye violet crystals, Lugol solution, ethanol 95%, safranin, media SIM, liquid medium MR-VP, TSIA media, ox bile salts, hydrogen peroxide, and hydrochloric acid.

2.2. Research Procedures

2.2.1. Analysis of Sugar Profile on Honey. Analysis of the sugar profile (glucose, fructose, sukrosa) on honey by using the AOAC HPLC 80:20 Acetonitril method: pure aqua water with a flow rate of 1ml/min detector RI (refractive index).

2.2.2. The Addition of Honey to Cassava Tapai-based Functional Beverages. Honey addition treatment with variations variations of 0% (control), 2%, 4%, 6%, and 8% into each functional Minimal sample to be analyzed at a later stage.

2.2.3. Isolation and identification of lactic acid bacteria. A total of 1 ml of the sample of a functional beverage of cassava (M. esculenta) tapai was resuspended into sterile aquades as much as 9 ml and homogenized. From the suspension is taken 1 ml, inserted into the diluent tube containing 9 ml sterile aquades then homogenized. Multi-level dilution is made from dilution of 10^-1 to 10^-7. Furthermore, 1 ml of the culture suspension in the last three dilutions (dilution 10^{-5}, 10^{-6}, 10^{-7}) was inoculated to the medium of MRS Agar with the technique of spread plate and incubated for 24 hours at a temperature of 37°C. Colonies of lactic acid bacteria are characterized by the presence of clear zones around the colony. The single colony formed was then selected and made a microbial stock culture in order to tilt for the next stage preparation.

2.2.4. Microbial characterization. Isolate characterization is carried out based on phenotypation characters including cell morphology, gram staining, motility, biochemical test (MR, VP, TSIA, and Cathalase test), as well as the potential test of probiotics (resistance to bile salts, resistance to acids, and resistance to temperature).
2.2.4.1. Gram staining. An isolate was put in the glass object, then fixated (plus 1 drop of aquaest, then dried anginkan/passed over a small flame), so that the stain was formed. On top of the stain is then put in succession: dye violet crystals, Lugol solution, ethanol 95%, and Safranin. Before setting the next solution, the suspension is left ± 1 minute, then rinsed with running water. Lastly, the glass object is closed with a glass deck and is observed under the Microkop. Observations were made against the shape and color of BAL. If the visible color is red, the isolates are Gram-negative, otherwise, if the visible color is purple, it means that the isolates include Gram-positive [4].

2.2.4.2. Motility test. The bacterial isolates are inserted into the semi-solid SIM media (Sulphide Indole Motility) in the reaction tube using a sterile puncture ose needle. It was incubated for 24 hours at 37ºC. Positive test is characterized by the growth of spread bacteria, the bacteria are moving (motil) and if the growth of bacteria does not spread only one line, then the bacteria are not moving (non motil) [5].

2.2.4.3. MR test. As many as 1 ose isolates were taken from the stock, then inoculated on the liquid medium MR-VP. Subsequently incubated for 5x24 hours at 37ºC temperature. After incubation, Methyl-Red is added by 5 drops above the bacterial isolating preparations. Positive results if the complex forms pink to red, indicating that the microbe produces acids [6].

2.2.4.4. Voges proskauer test. Bacterial isolates were taken as much as 1 ose and were inoculated into the MR-VP liquid medium then incubated at 37ºC for 3x24 hours. After incubation, then added 0.2 mL of KOH 40% and 0.6 mL of alfanaftol in each isolate and then shaken for 30 seconds. Positive results if the color of the medium changes from yellow to crimson [6].

2.2.4.5. TSIA test. One microbial isolate was taken and inoculated on the TSIA (Triple Sugar Iron Agar) medium by piercing the straight part of the butt and the Zigzag way in the slant (angled). Isolates were incubated at a temperature of 37ºC for 24 hours. The color changes that occur in the media are then observed. If the slanted part is red and the butt is yellow, the bacteria are able to ferment glucose, whereas when the slant and butt are both yellow, bacteria are able to ferment sucrose and duct.

2.2.4.6. Cathalase test. An isolate is applied to the glass object, then it is tested with H₂O₂ 3% and let stand for ± 1 minute. Positive trials are characterized by the formation of oxygen bubbles, indicating that the organism in question produces a catalosing enzyme that converts hydrogen peroxide into water and oxygen [4].

2.2.5. Probiotics Test

2.2.5.1. Resistance to bile salts. Synthetic bile salts (ox bile) are added to the MRSB medium with a concentration of 1% and 5%. Bacterial isolate is taken as much as 1 ose and is inoculated on the MRSB-salt medium. Isolates were incubated for 2-3x24 hours at a temperature of 37ºC. Positive results if marked with sedimentation on the base of the tube, and the change of media becomes more cloudy than before incubation [6].

2.2.5.2. Resistance to bile acids. Isolation of microbial isolates to stomach acid in the gastrointestinal tract low pH of about 2.5-3 is done by adding HCl 0.1 N to the MRSB medium. Bacterial isolates are taken from stock as much as 1 ose. It is then inoculated on the MRSB-HCl medium and incubated for 2-3x24 hours with a temperature of 37ºC. Positive results are characterized by the growth of bacteria in a medium that has low acidity (pH 2.5-3). Negative results if there is no microbial growth in the medium [6].
2.2.5.3. Resistance to bile temperature. Bacterial isolates were taken from the stock as much as 1 ose are then inoculated on the MRSB medium. Furthermore, the medium containing isolates was incubated at 15°C, 37°C, and 45°C for 2x24 hours. Then observed whether the bacterial plant occurs in the medium. Positive results in case of bacterial growth in the temperature range [4].

2.2.6. Molecular Test

2.2.6.1. DNA extraction. The DNA extraction of the genome is carried out by following the DNA extraction protocols contained in the Geneaid Presto™ Mini gDNA Bacteria Kit. First done sample preparation, bacteria as much as 1 x 10⁹ cells transferred to 1.5 ml tube microcentrifuge, then centrifuge for 1 minute with a speed of 14-16,000 x g then the supernatant discarded. Enter 200 μl gram (+) buffer onto tube and add lysozyme (4 mg/ml) and then vortex until Lysozyme is completely dissolved. Then enter a solution gram (+) buffer that has been added lysozyme into the sample then resuspension the cell pellets by using a vortex or a pipette. Incubation at 37°C for 30 minutes. During incubation do the reversal several times. After that, add 20 μl proteinase K then vortex to be mixed. Incubation at 60°C for 10 minutes, during incubation every 3 minutes, do a reversal.

Subsequent process samples were added 200 μl GB Buffer then vortex afterward incubated for 10 minutes at 70°C temperature. Next added 200 μl ethanol absolute then Divortex for 10 seconds then entered into GD Column and centrifuge with a speed of 14-16000 g for 2 minutes. Replace a collect tube with new one for that was added 400 μl W1 buffer then in the with speed 14-16000 g for 30sec. Then added 600 μl Wash Buffer than in the at 14-16000 G for 30 seconds, next the Column GD was moved into a sterile Eppendorf tube added 100 μl pre-heated elution after it was in centrifuge with a speed of 14-16000 g for 3 minutes. The Supernatant that is attached to the Eppendorf tube is DNA extract.

2.2.6.2. Amplification of DNA with PCR method. This procedure was done on a sample of an isolated DNA, previously created a reaction mixture for PCR i.e., 16 μl 2x Mytaq HS Red Mix and 2μl primary 16S rRNA Forward 63F respectively (5'-CAGGCCTAACACATGCAAGTC-3') and Primary Reserve 1387R (5'-GGGCGGTGTGTACAAGGC-3') and dH₂O 4 μl. The tubes then filled the PCR reaction mixture as much as 24 μl and then each tube was added 6μl of DNA extract. Amplification was carried out as many as 30 cycles and each cycle consisted of predenaturation of denaturation at 95°C for 5 min, at 95°C for 1 minute, annealing at 57°C for 1 minute, extending at 72°C for 1 minute and post extending at a temperature of 72°C for 10 minutes [7].

2.2.6.3. The PCR results are then visualized by electrophoresis. Results of electrophoresis in staining in etidium bromide for 15 minutes ago in destaining for 5 min. After that, visualization is done. Sequencing of 16S rDNA BAL is conducted in Macrogen Inc, South Korea. The 16S rRNA gene sequence obtained from the sequencing process is further analyzed using the Basic Local Alignment Search Tool (BLAST) program on the NCBI website to see the similarity with the reference sequence found in the Genebank. Furthermore, the phylogenetic tree is constructed based on the 16S rRNA gene sequence using the MEGA7 program with the Maximum Likelihood method.

2.2.6.4. Detection of PCR products with electrophoresis. Agarose Gel 1.5% made by mixing 1.5 gr of agarose powder into 100 ML TAE Buffer in Erlenmeyer was then heated into the microwave for 2 minutes to boil, then added 8 μl Ethidium bromide. The fluid gel is then cooled at room temperature. After a rather cold, the gel fluid is taken to the electrophoresis gel mold by using a gel comb with 14 wells of a comb. Each of the 5 μl of amplification products is inserted into the 1.5% agarose gel Well that is immersed in the tank containing TAE Buffer. Furthermore, Electrophoresis is run for 50 minutes with a constant voltage of 100 volts. After 50-minute electrophoresis is discontinued and the gel is lifted to be observed under UV rays. Positive result if there are DNA bands aligned with marker 198 BP and negative if there is no ribbon in the gel.
2.2.7. Data analysis
The analysis of sequencing results is done by performing BLAST nucleotide sequence of 16S rRNA sequencing results with databases available on the site www.ncbi.nlm.nih.gov. The result is then made by the phylogenies tree, with multiple alignment next, the visualization of kinship using the Neighbor-Joining tree with Bootstrap 1000x on MEGA Software (6.06) [8].

3. Results and Discussion

3.1. Honey Sugar Profile
The main components of honey sugar (monosaccharides, disaccharides, oligosaccharides), and water. Moisture and sugar content are important characteristics of honey, as water and sugar can affect appearance, texture, and flavor, as well as determining the durability of honey. The results of organic honey analysis used in research are described in Table 1.

| Component | Total |
|-----------|-------|
| Water     | 21.5% |
| Fructose  | 35.2% |
| Glucose   | 24.9% |
| Sucrose   | %     |
| pH        | 4.2   |

The results showed an organic honey water content of 21.5%. According to the National Standardization Agency of Indonesia (2013) [9], quality honey has a moisture content of 22% or less, so that the results of organic honey samples used to meet Indonesia's honey standard of 22%. The sugar levels in organic honey include fructose of 35.2%, 24.9% glucose, and 0% sucrose (undetectable) while the pH is 4.2. The results are slightly different from the data of the sugar content in honey obtained from the Beekeeping Center is 38.5% fructose, 31.0% glucose, and 1.5% sucrose while pH 3.9. Fructose and glucose are the highest sugar content in honey. The sugar content differs from the sugar content in granulated sugar, palm sugar, and palm sugar, in which 85-90% of its components are sucrose. The composition of honey is influenced by several things by the flower nectar that has been collected and issued by the bees that suck it, the climate factor and the maturity of honey.

3.2. Isolation and Characterization Lactic Acid Bacteria

3.2.1. Characterization. Characterization includes observations of cell morphology, gram staining, motility, biochemical tests (MR, VP, TSIA, and catalogic tests). The characterization results are presented in Table 2.

| Sample | Gram staining | TSIA | Catalase | Motility | MR | VP |
|--------|---------------|------|----------|----------|----|----|
| M1.A   | Bacil (-)     | RY   | -        | +        | +  | +  |
| M1.B   | Bacil (+)     | RY   | -        | +        | +  | +  |
| M2.A   | Bacil (-)     | Y    | -        | +        | +  | +  |
| M2.B   | Bacil (-)     | RY   | -        | -        | +  | +  |
| M2.C   | Bacil (+)     | RY   | -        | +        | +  | +  |
| M2.D   | Bacil (+)     | RY   | -        | +        | +  | +  |
| M3.A   | Bacil (-)     | Y    | -        | +        | +  | +  |
The result of characterization in Table 2 is that the gram coloration of 19 isolates in the form of basil and 1 isolate of cocus the red color, which means that the isolates are Gram-negative, otherwise if the visible color is purple means the isolates include Gram-positive.

The TSIA test shows 16 media that changed color to yellow red and 4 media changed color to yellow. This indicates that the media with red slant parts and yellow butt means that the bacteria are able to ferment glucose, while the media of the slant and butt are both yellow, meaning that the bacteria are able to ferment sucrose and duct.

The Cathalase test shows that 20 isolates of negative results mean that the bacteria do not produce a cataloging enzyme that converts hydrogen peroxide into water and oxygen.

The Motility test shows 16 positive isolates characterized by the growth of the spread bacteria, then the bacteria move (Motil) and 4 isolates the results are negatively characterized by the growth of bacteria does not spread in the form of one line, then the bacteria is not moving (non motil). The MR-VP test showed 20 isolates of positive results, indicating that the microbe produces acid and acetyl methyl karbinol (acetoin).

3.2.2. Probiotics test. A potential test of probiotics includes a temperature resistance test, resistance to acidity, and resistance to bile salts. The probiotic test results are presented in the following Table 3.

| Sample | Temperature (°C) | Acidity | Bile salt |
|--------|-----------------|---------|-----------|
|        | 15   | 37    | 45      |          |
| M1.A   | +    | -     | +       | -        |
| M1.B   | +    | -     | +       | -        |
| M2.A   | +    | +     | +       | -        |
| M2.B   | +    | +     | +       | +        |
| M2.C   | +    | +     | +       | -        |
| M2.D   | +    | +     | +       | +        |
| M3.A   | +    | +     | +       | -        |
| M3.B   | +    | -     | +       | -        |
| M3.C   | +    | +     | +       | -        |
| M3.D   | +    | +     | +       | +        |
A probiotic test on table 8 shows 20 isolates growing at a temperature of 15°C, 14 isolates grew at 37°C, and 20 isolates grew at a temperature of 45°C. While the acidity test shows, only 10 out of 20 isolates are grown on acidic conditions. In the bile salts test showed 15 out of 20 isolates grew in the condition. In general, there are 2 isolates of bacteria growing well on a resistance test against temperature, acidity, and bile salts that are M3D and M5B isolates. Both isolates were subsequently conducted molecular tests for the identification process of bacterial species.

3.2.3. Molecular Test. The molecular test is necessary to obtain the DNA profile of the organism identified. DNA extraction is an early process to obtain the DNA of an organism. DNA extraction starts from sample preparation until DNA extracts are obtained. DNA extraction was carried out on 2 isolates that had previously been isolated and conducted a potential test of probiotics from a functional beverage of cassava tapai that are M3D isolates and M5B isolates.

3.2.3.1. Visualization polymerase chain reaction result. In the DNA extraction phase of the two isolates of lactic acid bakeri extracted using the Geneaid Presto™ Mini gDNA Bacteria Kit. DNA extraction results were amplified by the PCR method using the Universal Gene 16S primary Forward 63F (5’-CAGGCCTAACACATGCAAGTC-3’) and the Primary Reserve 1387R (5’-GGGCCGTGTGTACAAGGC-3’), PCR amplification results visualized in electrophoresis and produced DNA bands aligned with marker 1,300 BP. PCR result is then obtained in-BLAST sequence to confirm the species.

Figure 1. Result of DNA amplification of samples with primers 63F and 1387R. M3D=isolate M3D; M5B=isolate M5B; M=Marker.

Both samples were M3D and M5B were amplified using the primary 63F and 1387R and showed a thick and bright ribbon. It demonstrates the sample DNA has a high concentration and indicates
amplification using a primary pair of 63F and 1387R. The results were in accordance with the research conducted by Barus et al. (2017) [7] who also used the primary pair of 63F and 1387R to amplify the gene sequence 16S rRNA produced a tape fragment of 1300 BP. Good quality DNA Extraction media products are indicated by DNA bands that look thick, clean, and lit.

3.2.3.2. Analisis of 16S rRNA Fragment by Sequencing. A DNA sequencing is aimed at determining the sequence of nucleotides: adenine (A), cytosine (C), guanine (G), thymine (T) in DNA molecules. The sequences obtained later compared to the sequential data contained in Genbank by doing the BLAST (Basic Local Alignment Search Tool). BLAST works with the principle of sequence alignment. BLAST performed on the site www.ncbi.nlm.nih.gov with a database of 16S ribosomal DNA sequences (bacteria and archaea). A DNA sequencing is performed on a "Single Pass DNA Sequensing" using the same primer as the 63F primary gene amplification. The identity of genes that have been known by the sequencer can be determined by comparing the similarity of data obtained with the sequential data contained in Genbank, one of which is NCBI. The sequencing result Data is further analyzed using blast nucleotides at www.ncbi.nlm.niv.gov. Blast result analysis provides information and verifies of organisms or bacteria that have a homologous DNA sample so that it can be used to know the identification of the bacteria. The acceptable percentage of sequencing data blast results is at least 95% unless the sequence data that is lower reading is applied at 75%.

| Isolate | Species Lactic Acid Bacteria Homolog          | Query coverage (%) | E value | Identities | Accession  |
|--------|---------------------------------------------|--------------------|---------|------------|------------|
| M3.D   | *Pediococcus acidilactis* Strain DMB.12     | 99%                | 0.0     | 98.14%     | MK748268.1 |
| M5.B   | *Weissella cibaria* strain MG5227           | 98%                | 0.0     | 98.56%     | MN368418.1 |

Based on the result of the blast data sequencing that the two isolated isolates are bacteria *Pediococcus Acidilactis* and *Weissella cibaria*, including lactic acid bacteria. *Pediococcus Acidilactici* is a Gram-positive species often found in pairs or tetrads. P. Acidilactici is a homofermentative bacteria that can grow in a wide range of pH, temperature, and osmotic pressure, thereby being able to colonize the gastrointestinal tract [10]. This species has emerged as a potential probiotic that has demonstrated promising results in animal and human experiments, although some results are limited. It is commonly found in fermented vegetables, fermented dairy products, and meats [11]. Pediococci is antagonistic towards other microorganisms, including enteric pathogens, mainly through the production of lactic acid and the secretion of bacteria known as Pediocins [12].

The *Weissella* species have been isolated from various sources, and some of them play an important role in fermentation. Weissella Cibaria was first described by Björkroth et al. And then found in various types of fermented foods.

4. Conclusion

Based on the results of research that has been done on the functional beverage of cassava (*M. esculenta*) tapai with the addition of honey treatment can be concluded as follows:

1. Honey added to the functional beverage of cassava (*M. esculenta*) tapai is genuine honey with water content and sugar sucrose in accordance with SNI.
2. The results of identification of lactic acid bacteria with the potential of probiotics namely *Paediococcus acidilactis* and *Weissella Cibaria*. 
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