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MOLECULAR IDENTIFICATION AND ANTIBACTERIAL ANALYSIS OF LACTIC ACID BACTERIA FROM COCONUT WATER (COCOS NUCIFERA) AS A PROBIOTIC CANDIDATE

Suryani Suryani[1,✉], Tuty Taslim[2], Ija Darmana[3], Rahmawati Rahmawati[4], Nova Arikhman[5], B.A. Martinus[6], Chrisnawati Chrisnawati[7] and Syamsuwirman Syamsuwirman[8]

[1]Department of Medical Laboratory Technology, Faculty of Health Sciences, Universitas Perintis Indonesia, Padang, Indonesia, 25586

[2]Department of Pharmaceutical, AKFAR (Akademi Farmasi Prayoga), Indonesia, 25144

[3]Department of Electrical Engineering, Bung Hatta University, Padang, Indonesia, 25133

[4]Department of Agrotechnology, Faculty of Agriculture, Muhammadiyah University of West Sumatera, Padang, Indonesia, 25171

[5]Department of Public Health, Faculty of Health Sciences, Baiturrahmah University, Padang, Indonesia, 25586.

[6]Department of Pharmacy, Indonesia Perintis University, 25586

[7]Department of Agrotechnology, Faculty of Agriculture. Universitas Maha Putra Muhammad Yamin, Indonesia, 23171

[8]Department of Agrotechnology, Faculty of Agriculture. Ekasakti University, Indonesia.

✉Corresponding Author: suryanimdiah@yahoo.com

ABSTRACT

Coconut water has been traditionally used as a fever medicine by the Minangkabau people in Indonesia since ancient times, but the chemical or probiotic components have not been widely studied. Therefore, this study aims to isolate and molecularly identify the probiotics found in coconut water, as well as to analyze their antimicrobial and antioxidant abilities. The Lactic acid bacteria (LAB) isolation was conducted by applying MRSA + CaCO3 with a concentration of 0.5%, and the molecular identification used the PCR (Polymerase Chain Reaction) method. Meanwhile, morphological and physiological identifications were also carried out with biochemical tests. The agar diffusion method was used to analyantimicrobialsial by measuring the diameter of the inhibition zone. The results showed that 97 isolates were lactic acid bacteria, while morphological, physiological, and biochemical tests showed *Lactobacillus paracasei*, *Lactobacillus plantarum*, *Pediococcus sp* 1, *Pediococcus sp* 2, *Pediococcus sp* 3, and *Pediococcus sp* 4, as well as two species of fungi such as *Candida sp* and *Rizhopus*. The molecular identification showed that *Enterococcus faecalis* strain 2358 can inhibit the growth of *Staphylococcus aureus*. Therefore, coconut water can be traditionally used to treat fever medicine because it contains probiotics and antimicrobial properties.

Keywords: Molecular Identification, Probiotics, Coconut Water, Enterococcus Faecalis Strain 2358, Lactic Acid Bacteria

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INTRODUCTION

Coconut water (Cocos nucifera) has been used by the Minangkabau people in Indonesia as a supplement drink. The fresh preparation can serve as a remedy for sick adults and children. Some previous research, such as[1], studied important elements, such as Fe, Mn, Zn, and Cu. Furthermore, the development of coconut water as a functional drink was also investigated[2] by fermentation and the addition of lactic acid bacteria *Lactobacillus rhamnosus* SP l. Fermentation lasted five days, and the findings revealed that the drink's quality was good, with lactic acid bacteria levels reaching 82 × 108 CFU / mL L. Instead of the benefit, fermentation of coconut water can be toxic if it is contaminated by the fungus Arthrinium.[3] The reason is that this fungus contains lipophilic and 3-nitro propionic acid which is fatal to be consumed. It blocks the

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citric acid cycle in cells. The symptoms are similar to bongkrekic acid poisoning. Additionally, coconut water affects rats' blood sugar and retina.[4,5] In diabetic patients, it can also reduce blood sugar levels and repair the retina. Some studies have been conducted on its micro-element content, but not many have isolated lactic acid bacteria in fresh coconut water. Therefore, Molecular Identification and Antimicrobial Analysis of Lactic Acid Bacteria need to be carried out. The presence of this eliminates the requirement for a starter to produce functional drinks from fermented coconut water.

EXPERIMENTAL

Sample
The sample for identification and isolation was bacterial isolate and fresh coconut water, diluted with saline to a 10-5.

Chemicals
The chemicals used were MRS (De Man Rogosa Sharp, Merck, Germany) Broth, MRSA, SIM (Sulfide Indole Motility) media, CaCO3 (Merck), sterile NaCl, sterile Aquades, NA broth media, agarose, fresh coconut water, Mc Farland standard solution 0.5 and 96% alcohol, H2O2, iodine solution, Violet crystal solution, Safranin solution, Chelex 10%, Mc Farland 0.5.

Instruments
The tools used included UV-Vis Spectrophotometry (Molyneux, 2004), Olympus CX33 Microscope, Laminair Air Flow (Class 100 | B-One Messgerate 915 S), Autoclave GEA LS-B75 L, Vortex Mixer VM - 300, glassware such as Erlenmeyer, Petri dishes, test tubes, glass beakers, as well as cotton, filter paper, ose, aluminum foil, glass rods, and Socorex micropipettes

The Lactic Acid Bacteria Isolation from Coconut Water
About 100 ml of fresh coconut water was diluted using sterile saline or NaCl to a dilution of 10-5. Meanwhile, a selective medium was prepared in the form of 0.5% MRSA + CaCO3. About 1 ml of coconut water in each dilution was pipetted into the media in the petri dish by pour-plate and was incubated overnight at 370C.

Morphological Identification of Lactic Acid Bacterial Isolates
Bacterial isolates growing in the "Halo" area were taken for 1 ose and scratched on MRSA media until a single colony was obtained. This colony was tested for Gram staining to determine cell shape, colony shape, colony color, cell surface shape, and catalase biochemical test.

Gram Stain Test
One ose isolate of lactic acid bacteria was prepared on a glass object, then fixed on a Bunsen fire. Furthermore, the preparation was dripped with a crystal violet solution, left for 1 minute, and dried after it was washed with running water. An iodine solution was dripped on a glass object and left for 2 minutes. Next step, it was washed again with running water and dried. Subsequently, a drop of alcohol was added until the purple color disappeared. Safranin was then added, remained for 30 seconds, washed with running water, and dried. Therefore, the preparation was perceived under a microscope.

Catalase Test
One ose isolate of lactic acid bacteria was taken and smeared on a glass object. A drop of H2O2 solution was then added to the glass object with a concentration of 3% to form gas bubbles during the preparation.

Motile Test
This motile test was carried out using SIM (Sulfide Indole Motility) or upright media. One ose isolate from MRSA media was taken and inserted into semi-solid media (upright SIM media), then incubated for 48 hours at 37ºC. A positive result showed several patches around the ose needle puncture mark on the medium.

Sample Preparation
For colonies of lactic acid bacteria (procaryotes) (16S rDNA), DNA was extracted from the sample using the GES method (Pitcher et al. 1989 Modified) as follows. First, the purified colonies were placed into an

Eppendorf tube containing 250 µl of 10% Chelex. The colonies were homogenized using Vortex for 10 seconds and centrifuged at 15,000 rpm for 30 seconds using a microcentrifuge. Subsequently, the colonies were incubated at 950C for 30 minutes with a heating block. They were later vortexed for 30 seconds and centrifuged at 15,000 rpm. Quality control (QC) was then carried out and continued with PCR.

The PCR Steps are as Follows

DNA Amplification (PCR)

The amplification was performed using go-Taq Master Mix (Promega) and primer:
For procaryotes (16S rDNA):
27 F: 5`-- AGA GTT TGA TCC TGG CTC AG – 3`
1492R: 5`-- TAC GGY TAC CTT GTT ACG ACT T -–3`

Sequencing

For Sanger Sequencing, The Primers Used Are:
For Procaryotes (16S rDNA):
Primer 518 F: 5' – CCA GCA GCC GCG GTA ATA CG – 3'
Primer 800 R: 5' – TAC CAG GGT ATC TAA TCC –3'
Primer 1492R: 5`-- TAC GGY TAC CTT GTT ACG ACT T -–3`
Genetic Analyzer ABI 3130 XL conducted Sanger sequencing

Antimicrobial Activity Analysis

Antimicrobial analysis was carried out utilizing Staphylococcus aureus, Escherichia coli, Listeria monocytogenes, and Salmonella typhi. The test bacteria were rejuvenated by scratching on NA agar media. The bacterial colonies were collected one at a time and placed in a test tube containing NA broth, then incubated overnight. Furthermore, 1 µl of this bacterial culture was taken and put in a test tube containing sterile distilled water, then homogenized and compared with 0.5 Mc Farland standard solution. Using an L glass rod, 10 µl of the solution was levelled on an NA agar substrate produced in a Petri dish plate. The Lactic Acid bacterial isolate to be tested had been rejuvenated in a test tube, and 10 µl was taken and placed in a sterile container. The filter paper shaped into a sterile circle was dipped in the isolate until the isolate ran out. Subsequently, it was placed in a Petri dish that had been spread with the test bacteria. Incubation was then conducted overnight at 370C, and the diameter of the Hallo area was measured.

Standard Mc Farland 0.5 Preparation

Mc Farland's standard solution is a mixture of 1% BaCl2 and H2SO4 with a composition of 0.05 ml and 9.95 ml. The standard solution was homogenized, and the turbidity was measured with a spectrophotometer at a wavelength of 620 nm. Distilled water was used as a blank, and the absorbance value was 0.08 to 0.13. Therefore, this Standard Mc Farland solution was equivalent to cell suspension (cell count/cell concentration) of 1.5x108 CFU/mL.

RESULTS AND DISCUSSION

The LAB Isolation from Coconut Water

The result of this isolation, using MRSA + 1% CaCO3 selective media, obtained 97 isolates, as shown in Table-1 below:

Table-1: Results of Isolation

No	[Sample]	Petri Number	Number of Colonies
1.	10^{-5}	3	6
2.	10^{-6}	5	38
3.	10^{-7}	5	53
	Sum		97

Based on Table-1, more colonies of lactic acid bacteria were obtained at a larger sample dilution. In a more dilute sample, the density of colony growth gets smaller, and the colonies are freer to grow. Meanwhile, the growing colonies will be crowded together at large sample concentrations or small dilutions, and they cannot be taken. Lactic acid bacteria create acid during their development. The addition of $CaCO_3$ to an alkaline MRSA medium triggers a neutralization reaction, resulting in a clean zone surrounding the expanding bacterial colonies. The addition of $CaCO_3$ to an alkaline MRSA medium triggers a neutralization

reaction, resulting in a clean zone surrounding the expanding bacterial colonies. Therefore, these bacteria are in the center of the clear zone or the "Hallo" area. This is consistent with research on the isolation from grouper[6] where the media used was also added with $CaCO_3$.[5] Meanwhile, this is different from the research conducted on the isolation from grasshoppers' intestines, a new probiotic candidate for digesting cellulose.[1] The medium used was MRSB (de Mann Rogosa Sharp Broth) plus 1% *Carbonmethylcelluso* (CMC) in this research. In isolating lactic acid bacteria from fermented meat in China, the medium used was MRS Broth.[7] Colonies on MRSA or MRS Broth media and those growing on MRSA + CaCO3 had differences. Colonies growing on MRSA media were round, raised, and yellowish, while those on MRSA + Ca CO3 media grew in the middle of the clear zone. Moreover, colonies developing on MRS medium should be identified using the Analytical Profile Index (API) 50 CH kit.[8]

Morphological Identification of LAB Isolates

The acid bacteria were recognized morphologically by microscopic examination of the cell shape, colony shape, colony color, and cell surface shape of the 97 colonies. Biochemical tests were also carried out, and the results can be seen in Table-2 below.

Table-2: LAB Morphological Test Results from Fresh Coconut Water

Number of Isolates	Gram	Cell Shape	Colony Shape	Colony Color	Elevation	Catalase	Motile	Description
12	+	rod	small round	white	raised	-	-	*Lactobacillus paracasei*
37	+	rod	small round	white	raised	-	-	*Lactobacillus plantarum*
14	+	coccus	tetrad	cream	flat	-	-	*Pediococcus* sp 1
19	+	coccus	tetrad	cream	flat	-	-	*Pediococcus* sp 2
21	+	coccus	tetrad	cream	flat	-	-	*Pediococcus* sp 3
14	+	coccus	tetrad	cream	flat	-	-	*Pediococcus* sp 4

Based on Table-2, the Gram test showed that all isolates were Gram-positive. The cell morphology revealed that 49 cells were rod-shaped, and the remainder were coccus-shaped. The majority of the colonies were white, with the remainder being cream. Catalase and motility assays yielded negative results in biochemical studies. The Bergey's Manual of Systematic Review showed that the isolates consisted of 6 species such as *Lactobacillus plantarum*, *Lactobacillus brevis*, *Pediococcus* 1, *Pediococcus* 2, *Pediococcus* 3, and *Pediococcus* 4.[9] This is consistent with the morphology of LAB isolated from pickles and has potential as probiotics[5,10,11] and morphology of pathogenic bacteria.[12]

Molecular Identification

The molecular identification result is the DNA sequence, as shown in Fig.-1. This DNA sequence is processed to construct a phylogenetic tree, as seen in Fig.-2. Based on Fig.-2, the molecularly identified lactic acid bacteria were *Enterococcus faecalis* strain 2358. PCR also investigated similar lactic acid bacteria identification[13] stating that DNA sequences were created by both lactic acid bacteria from distinct sources, and the identification result was *Lactobacillus johnsonii* 456. The isolates from Teff fermentation were also identified molecularly by PCR, and the phylogenetic tree was made.[14] Molecular identification by PCR using 16SrRNA was also used to identify other bacteria found in the intestine[15] and the phylogenetic tree was also made. It can also be informed that fresh coconut water contains lactic acid bacteria. In contrast to research that used coconut water as a functional drink[2] added lactic acid bacteria of *Lactobacillus rhamnosus*.

Antimicrobial Activity Analysis

The antimicrobial analysis uses test bacteria such as *S. aureus, E. coli, L. monocytogenes*, and *S. typhi*. The results can be analyzed by measuring the inhibition zone diameter of the bacteria, as shown in Table-3 below:

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CGCCCTCCTTTCGAGCCTCAGCGTCAGTTACAG
ACCAGAGAGCCGCCTTC
GCCACTGGTGTTCCTCCATATATCTACGCATTT
CACCGCTACACATGGAA
TTCCACTCTCCTCTTCTGCACTCAAGTCTCCCA
GTTTCCAATGACCCTCC
CCGGTTGAGCCGGGGGCTTTCACATCAGACTTA
AGAAACCGCCTGCGCTC
GCTTTACGCCCAATAAATCCGGACAACGCTTGC
CACCTACGTATTACCGC
GGCTGCTGGCACGTAGTTAGCCGTGGCTTTCTG
GTTAGATACCGTCAAGG
GATGATCAGTTACTAACGTCCTTGTTCTTCTCT
AACAACAGAATTTTACG
AACCGAAAACCTTCTTCACTCACGCGGCGTTGC
TCGGTCAGAATTTCGTC
CATTGCCGAAAAATCCCTACTGGTGCCTCCCGT
AAGAATTTGGGCCGTGT
CTCAATCCCAATGTGGCCGAACACCCTCTCAGG
TCGGCTATGCATCGTGG

Fig.-1: The DNA Sequence of Identified Lactic Acid Bacteria from *Pediococcus*

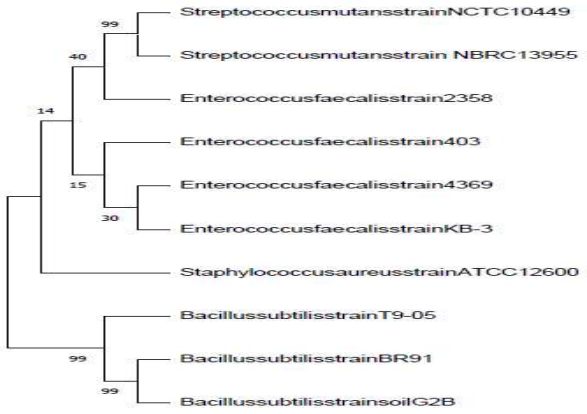

Fig.-2: Phylogenetic Tree of LAB from *Pediococcus*

Table-3: The Analysis of Antibacterial Activity

Lactic Acid Bacteria (LAB)	Sample	*Escherichia coli* (mm)	*L. monocytogenes* (mm)	*Salmonella. typhy* (mm)	*S. aureus* (mm)
Enterococcus faecalis strain 2358	Pl 4.1	24	26	23	27
	Pl 4.4.	26	27	25	22
	Pl 4 .7	25	23	23	24
Average		25	25.3	23.6	24.3

Based on Table-3, the isolates from fresh coconut water can inhibit the growth of the test bacteria. *Enterococcus faecalis* strain 2358 can inhibit the growth of *L. monocytogenes* and *E. coli* with the largest inhibition zone diameter of 25.3 mm and 25 mm. Meanwhile, it can inhibit *S. typhi* with the smallest inhibition zone diameter of 23.6 mm. The growth of all test bacteria can be inhibited with a large diameter.

This analysis of antimicrobial ability is similar to Nissin against gram-negative bacteria.[16–18] Another study was carried out on the microbiota as the source of the new antimicrobials.[19] This was also conducted when analyzing antimicrobials from VCO against *S. aureus*.[20]

CONCLUSION

Based on the findings, it is normal for the Minangkabau people of Indonesia to consume coconut water as a supplement drink when they have a fever. This is because coconut water contains lactic acid bacteria of *Enterococcus faecalis* strain 2358 and has a good antimicrobial ability.

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CONFLICT OF INTERESTS

The authors declare that there is no conflict of interest.

AUTHORS CONTRIBUTION

All the authors contributed significantly to this manuscript, participated in reviewing/editing, and approved the final draft for publication. The research profile of the authors can be verified from their ORCID ids, given below:

Suryani Suryani https://orchid.org/0000-0001-6669-6934
Tuty Taslim https://orchid.org/0009-0001-2698-9107
Ija Darmana https://orchid.org/0009-0001-1108-7469
Rahmawati Rahmawati https://orchid.org/0009-0007-0096-3877
Nova Arikhman https://orchid.org/0009-0008-5876-1706
B.A. Martinus https://orchid.org/0009-0000-2789-8242
Chrisnawati Chrisnawati https://orchid.org/0009-0004-5193-4353
Syamsuwirman Syamsuwirman https://orchid.org/0009-0001-4810-7507

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