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# ISOLATION AND MOLECULAR IDENTIFICATION OF LACTIC ACID BACTERIA (LAB) IN COCONUT MILK FERMENTATION

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## ABSTRACT

Lactic Acid Bacteria (LAB) contains of bacteriocin which is peptide that has the capacity to isolate the growth of pathogenic bacteria, where in contrast is harmless for other good bacteria. LAB is found in material fermentation containing high carbohydrate and protein like coconut milk which is undergone the process becoming Virgin Coconut Oil (VCO). In the fermentation process, there were three layers formed; oil, blondo, and water (waste). The LAB isolation on coconut milk fermentation used MRSA + 0,5% $CaCO_3$ as the selective media. with the dilution from $10^{-1}$ to $10^{-7}$. Here, each sample was taken from each layer formed in the milk fermentation process. The identification was carried out in two ways, first was morphology identification, and the second one was molecular identification applying the PCR method. There were 97 isolates obtained from oil layer, 23 isolates from Blondo layer, and 14 isolates from water layer. After being identified well based on both morphology, and molecular on the oil layer, there were six LAB found, which were *Lactobacillus paracasei, Lactobacillus plantarum, Micrococcus luteus, Corineaebacterium bovis, Lactobacillus thermobacterium* dan *Corineaebacterium xerocis.* Three types of LAB within the blondo were identified as *Lactobacillus plantarum, Lactobacillus paracasei* and *Lactobacillus thermobacterium.*

**Keywords:** Isolation, molecular identification, Virgin Coconut Oil (VC)_, Lactic Acid Bacteria (LAB), PCR


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**ABSTRAK**

Bakteri Asam Laktat (BAL) mengandung bakteriosin yaitu peptida yang mempunyai kemampuan menghambat pertumbuhan bakteri patogen, tetapi tidak berbahaya bagi bakteri baik. Bakteri asam laktat terdapat pada fermentasi bahan yang mengandung karbohidrat dan protein tinggi seperti santan diproses menjadi Virgin Coconut Oil. Pada proses fermentasi santan menjadi Virgin Coconut Oil (VCO) terbentuk tiga lapisan yaitu lapisan Minyak, lapisan Blondo dan lapisan Air (kotoran). Isolasi Bakteri Asam Laktat dari fermentasi santan menggunakan media selektif MRSA + 0,5% $CaCO_3$ dan media MRSA saja dengan pengenceran $10^{-1}$ sampai $10^{-7}$. Dimana sampel diambil dari setiap lapisan hasil proses fermentasi santan menjadi VCO. Identifikasi dilakukan dengan dua cara yaitu identifikasi morfologi dan identifikasi molekular dengan menggunakan metoda PCR. Dari lapisan Minyak didapat 97 isolat, lapisan blondo 23 isolat dan lapisan Air 14 isolat. Setelah diidentifikasi baik secara morfologi maupun molekular ternyata pada lapisan minyak terdapat 6 jenis Bakteri Asam Laktat (BAL) yaitu *Lactobacillus paracasei, Lactobacillus plantarum, Micrococcus luteus, Corineaebacterium bovis, Lactobacillus thermobacterium* dan *Corineaebacterium xerocis.* Pada blondo didapatkan 3 jenis Bakteri Asam Laktat (BAL) yaitu *Lactobacillus plantarum, Lactobacillus paracasei* dan *Lactobacillus thermobacterium.*

**Kata kunci:** isolasi, identifikasi molekular, Virgin Coconut Oil(VCO), Bakteri Asam Laktat (BAL), PCR

## INTRODUCTION

Lactid acid bacteria (LAB) are bacteria isolated from materials which are rich mainly in carbohydrates and containing high protein [1] and are able to ferment those material in order to produce lactid acid. These bacteria are beneficial as source of probiotic [2],[3],[4] and contain of bacteriocin [5],[6] which is peptides that can destroy the wall of patogen bacteria cell and kill those bacteria, which is in contrast to good bacteria.

Bacteriocin has huge potential as food preservative [7], [8] besides its ablility as antimicrobial [9], [10] which in this work proves that the bacteriocin existing in lactid acid *Lactobacillus* spp can inhibit the growth of *chloramfenikol*, *Ampisilin* and *Tetrasiklin* antibiotics. In general, LAB isolation is in line with the observation of its antibacteria [10], [11], [12].

The ability of LAB containing bacteriocin which function as either antimicrobial or antibiotics is equally important with the ability of natural antibiotics which are isolated from the plants like *Trichomanes chinense* [13], and is able to inhibit *Staphilococcus aureus, Salmonella typhimurium,* and *Escherichia coli*. Other antibiotics derived from Marine Actinomycetes microbe which is *Sterptomyces sp* A11 has been determined as well [14]. Apparently, this compound can also inhibit *Bacillus substillis, E.coli,* and *Pseudomonas aeruginosa*.

Several of LAB have been isolated from various sources like in Turkey [11] obtaining 45 LAB isolates which are isolated from “Boza” sample, and consisted of *Lactococcus lactis*


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subsp, *Leuconostoc citreum, Lactobacillus brevi, Lactobacillus plantarum, Lactobacillus paraplantarum, Enterococcus faeciu, Lactobacillus graminis, Pediococcus species and Lactobacillus paracasei* subsp. *paracasei*. The LAB of Medicinal herbs originated from Pirandai have also been isolated [15], and *Lactobacillus acidophillus* and *Lactococcus rafinolactis* were obtained. Traditional fermented food originated from West Sichuan Area like yoghurt is also containing LAB [16] namely *Lactobacillus* and *Lactococcus*, which is further identified applying PCR, and turned out as *Lactobacillus fermentum* and *Lactococcus lactis.* Fermented food from other areas like "Teff" contains LAB [12] such as *Lactobacillus brevis, Lactobacillus paracasei* and *Enterococcus faccium.* Besides that Dairy fermented food is also included into those which contain lactid acid bacteria *Lactobacillus* spp [10]. According to what have been mentioned above, the lactid acid bacteria isolation have been performed from various material [2] i.e., cheese, kefir grains, milk, beverage, source of poultry, cow rumen fluids, human feces, chicken feed, beef dadih, pineapple waste, and etc.

Nevertheless, there is none performed in isolating the lactid acid bacteria of the coconut milk fermentation process forming Virgin Coconut Oil (VCO). The fermented coconut milk producing VCO has many advantages such as losing weight, reducing cholesterol level, and inhibiting the groth of pathogenic bacteria, or fucntioning as both antibacteria and antivirus [17], [18], [19].

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## EXPERIMENTAL SECTION

This research was conducted at several laboraties, i.e., LLDIKTI Region X Laboratory, Baso Veterinary Laboratory, Biomedical Laboratory, Medical Faculty of Andalas University. and Chemistry Laboratory of Muhammadiyah University of West Sumatra.

### Materials

The materials used in this research were as follows: Coconut milk which was processed into Virgin Coconut Oil, oil layer (VCO), Blondo layer and water layer obtained from the process of making VCO through coconut milk fermentation. Whereas the media taken for isolating Lactic Acid Bacteria were Mannosa Rogosa Sharpe Broth / MRSB (Merck), CaCO3 (technical), Agarosa (Merck). In addition, sterile saline solution, MRSA and MRSA + 0.5% CaCO3 were used as the ingredients to identify morphologically, and to perform biochemical tests such as Complex Iodine, Safranine, Alcohol, Aquades and so on. Whilst the materials used for molecular identification were primary, 500µl Tris EDTA (TE), ammonium acetate, SDS-Polyacrylamide (SDS-PAGE) gel material 18-20%, comassife blue for. Ase RNA 3 µl, 70% ethanol, lysozyme, obtained ddH2O 27 µl, phenol, SDS (Sodium Dodesil Sulfate), chloroform, Proteinase K (10 mg / µl), isoamil alcohol 25: 24: 1, and protein marker with the

size of 10,000-40,000. Typically, DNA α, Tris HCl pH 8, isopropanol to fractionate DNA, ethidium bromide, agaros gel, 3M acetate, agarose, TBE buffer (Tris-Boric-EDTA) were taken.

**Instrumentation**

Here in this research, the equipment used in order to isolate and to conduct morphology identification as well as to perform biochemical tests of Lactic Acid Bacteria besides those commonly used glass tools were Autocklaf, Laminar Flow and Microscopes. In another hand, for molecular identification Electrophoresis was also used other than PCR.

**Procedure**

Isolation of Lactic Acid Bacteria

In order to get isolates, BAL was isolated from three existing layers containing coconut milk fermentation process to become Virgin Coconut Oil, namely oil layer, blondo layer and water layer. Isolation was carried out using 2 media, they were MRSA media and MRSA + media 0.5% CaCO3. By applying a dilution method up to 10-7, the isolation process was performed for several times using the pour plate and streak plate method, so that a number of isolates could be obtained which was followed by the identification process morphologically along with biochemical tests.

**Morphological identification**

Then the obtained isolates were proceeded by performing identification morphologically where isolates were planted in MRSA media, and incubated at 37 0 C. Observed on the shape of the colony, some were convex, whereas some others were flat or concave. Examined also the color of the colonies, where there were white in color, yellow, yellowish or clear and so on. The arrangement of cells were also significant to be taken into account whether the shape of the cell was round or hollow.

Biochemical tests

The conducted biochemical tests were Catalase test, carbohydrate fermentation, oxidase, ammonia production (NH3), and TSIA test, according to Mac Faddin (1983) procedure, then compared them to the manual (Cowan, 1975)

**Molecular identification**.

Molecular identification was initiated with the genomic isolation stage of lactic acid bacterial DNA isolates obtained from morphological identification and biochemical tests, then 16S rRNA gene amplification PCR 16S rDNA amplification per reaction of 30 µL using 27F


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primer (5'-AGAGTTTGATCCTGGCTCAG-3 ') position 8-27 on the E. Coli chromosome and primer 1492R (5'-GGTTACCTTGTTACGA CTT -3 ') in positions of 1510-1492 on the E. coli chromosome (Nikolova et al. 2009), followed by analyzing it in the Electrophoresis Gel and was ended by performing its sequencing data analysis.

## RESULT AND DISCUSSION

### Isolation of Lactic Acid Bacteria

The colony of Lactic Acid Bacteria isolation using the MRSA media in the absence of adding 0,5% $CaCO_3$ was able to be measured at the dilution of $10^{-5}$ up to $10^{-7}$. At $10^{-1}$ up to $10^{-4}$ dilution, the colonies were grown so dense therefore they were unmeasureable.The grown bacteria were yellowish, convex, and rather shiny. How ever, as highlighted by Delfaedah and Sumaryati Syukur (2013); Hoque (2010), Heravi (2011), Syukur and Husmaini (2014) that those grown colonies were identical among each other, and there were co certainty that these were LAB colonies. To confirm that these were LAB colonies, Husmaini and Endang Purwati (2012) suggested to proceed using KIT AOI CHL 50. The LAB colonies which were grown in MRSA media can be seen in Figure 1 below:

Figure 1. LAB isolates colony grown in MRS media

LAB isolation process using MRSA media + 0,5% $CaCO_3$, would form the "Halo" area, where one colony was found in its center, and was confirmed as LAB bacteria colony. $CaCO_3$ would be reacted with the acid produced by LAB, neutralized it, and made the area free from bacteria and in clear condition. The isolates grown in the center of "Halo" area were picked using ose, and then scratch them to MRSA media for morphology identification. LAB colony grown in MRSA media + 0,5% $CaCO_3$ can be seen in Figure 2. below:


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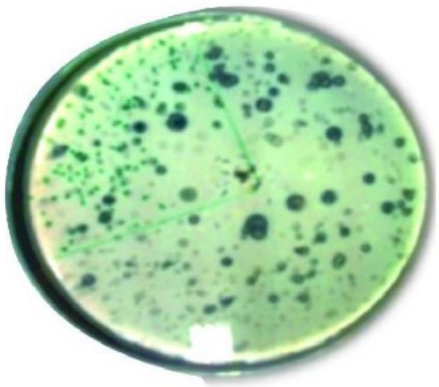

Figure 2. LAB isolate colony grown in MRSA media + 0,5% $CaCO_3$

The use of MRSA media + 0,5% $CaCO_3$ in isolating LAB was in accordance as highlighted by Rukmini Putri (2012); where she fermented Lactid Acid Bacteria obtained from fermentation of *Growol*, Indonesia traditional cuisine, by using MRSA media added with 1,5 % $CaCO_3$ . Whereas, Nguyen (2010_, isolated LAB using MRSA media 1 % $CaCO_3$ as well on the Vietnamese traditional food namely *Nem chua*, and Sarkono (2010) isolated the LAB derived from abalone using MRSA media + 0,5 % $CaCO_3$, the indicated colony was the growth of LAB bacteria marked by the clear zone in its surrounding.

The LAB isolate colony after being measured is shown in Table 1 below:

**Table 1. LAB isolates amount of isolated result**

| No. | Layer | Isolate Amount |
|---|---|---|
| 1. | Oil | 97 |
| 2. | Blondo | 23 |
| 3. | Water | 14 |

**Morphology Identification**

There were 134 LAB isolates of the isolation result using the MRSA media + $CaCO_3$ would experience further analysis process on morphology identification. It resulted variation in shape of colony like convex, flat, and concave, variation in color like white, yellow, yellowish, and clear, and variation in smell like odour and odourless. This morphology identification result of LAB isolates was confirmed by biochemical test's results such as gram staining, motility test, and others, and combined with physiology test as shown in Table 2 below:


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**Table 2. Result of Morphology Identification on LAB Isolates**

| No. | Layer | Type of LAB |
|---|---|---|
| 1. | **Oil Layer** | *Lactobacillus plantarum*<br>*Lactobacillus paracasei*<br>*Micrococcus luteus*<br>*Corineaebacterium bovis*<br>*Corineaebacterium xerosis*<br>*Lactobacillus thermobacterium* |
| 2. | **Blondo Layer** | *Lactobacillus plantarum*<br>*Lactobacillus paracasei*<br>*Lactobacillus thermobacterium* |
| 3. | **Water Layer** | *Micrococcus luteus*<br>*Corineaebacterium bovis*<br>*Corineaebacterium xerosis* |

**Molecular Identification**

From 134 LAB isolates obtained, some were performed further identification molecularly using PCR, in order to determine their LAB types, and to find out the sequence of its LAB DNA. The molecular identification was initially conducted onto four isolates. Its analysis result using the PCR composition and profil was pointed out in the Figure 3 below:

**Komposisi PCR :**

| Reagent | Volume |
|---|---|
| Go Taq Green Master Mix (Promega) | 12,5 µl |
| Primer Forward 16sRNA | 1 µl |
| Primer Reverse 16sRNA | 1 µl |
| DNA | 1 µl |
| Nuclease Free water | 9,5 µl |

**Note : DNA → 1 koloni tunggal dicuplik kemudian dilarutkan kedalam 20 µl Nuclease Free water**

**Profil PCR :**

| | | | |
|---|---|---|---|
| Denaturasi | 95C | 3 menit | |
| Denaturasi Awal | 95C | 30 detik | 35 Siklus |
| Annealing | 60C | 30 detik | |
| Elongasi | 72C | 30 detik | |
| Elongasi Akhir | 72C | 5 menit | |

**Figure 3. PCR Composition and Profile**


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Produced electropherogram as shown in Figure 4 below:

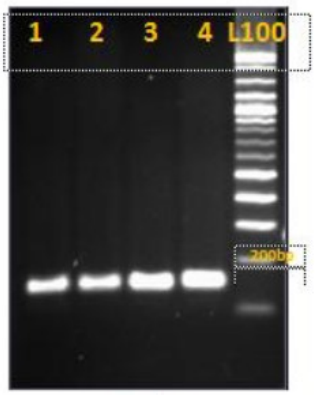


**Figure 4.** Electropherogram on PCR Result using **16s RNA Primer**

Its sequencing is highlited in the Figure 5 below:

1. **Bac1**

   **Forward :**

   GACGTCCCATGAGAGTTTGTACAGCCGAAGCCGGTGGCCTAACCTTTTTGGGGGAGAGCCCC

   CTAAAGCGTGAGACATGAGAGGGGGGGAGATCTCATAAAGGTGTCCGTAAAA


**Figure 5. Result of Isolate No 1 Sequencing**

In the figure shown above, the sequencing result on LAB No 1 isolate is not detected.

The following is Figure 6. The isolate No 2 sequencing result.


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2. **Bac2**

**Consensus :**

CGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGAGAGTTTGTAACACC
CAAAGTCGGTGGGGTAACCTTTTAGGAACCAGCCGCCTAAGGTGGGACAGATGATTAGGG
TGAAGTCGTAACAAGGTAGCC

**Sequences producing significant alignments:**

Select: All None Selected:0

Alignments Download GenBank Graphics Distance tree of results

| Description | Max score | Total score | Query cover | E value | Ident | Accession |
|---|---|---|---|---|---|---|
| Lactobacillus plantarum strain GB-LP1, complete genome | 265 | 1325 | 100% | 7e-68 | 100% | CP020564.1 |
| Lactobacillus plantarum strain dm, complete genome | 265 | 1325 | 100% | 7e-68 | 100% | CP022373.1 |
| Lactobacillus plantarum subsp. plantarum strain CGMCC 1.557, complete genome | 265 | 1325 | 100% | 7e-68 | 100% | CP016270.1 |
| Lactobacillus pentosus strain SLC13, complete genome | 265 | 1325 | 100% | 7e-68 | 100% | CP022130.1 |
| Lactobacillus plantarum strain LPL-1, complete genome | 265 | 1325 | 100% | 7e-68 | 100% | CP021997.1 |
| Lactobacillus plantarum strain VJC38 16S ribosomal RNA gene, partial sequence | 265 | 265 | 100% | 7e-68 | 100% | KP144784.2 |
| Lactobacillus plantarum subsp. plantarum isolate SRCM100434, complete genome | 265 | 1325 | 100% | 7e-68 | 100% | CP021528.1 |
| Lactobacillus plantarum strain SRCM102022, complete genome | 265 | 1325 | 100% | 7e-68 | 100% | CP021501.1 |
| Lactobacillus plantarum strain P4 16S ribosomal RNA gene, partial sequence | 265 | 265 | 100% | 7e-68 | 100% | MF098786.1 |
| Lactobacillus plantarum strain TMW 1.1623, complete genome | 265 | 1325 | 100% | 7e-68 | 100% | CP017379.1 |
| Lactobacillus plantarum strain TMW 1.708, complete genome | 265 | 1325 | 100% | 7e-68 | 100% | CP017374.1 |
| Lactobacillus plantarum strain TMW 1.277, complete genome | 265 | 1320 | 100% | 7e-68 | 100% | CP017363.1 |
| Lactobacillus plantarum strain TMW 1.25, complete genome | 265 | 1320 | 100% | 7e-68 | 100% | CP017354.1 |

Sequencing result on Isolate No 2

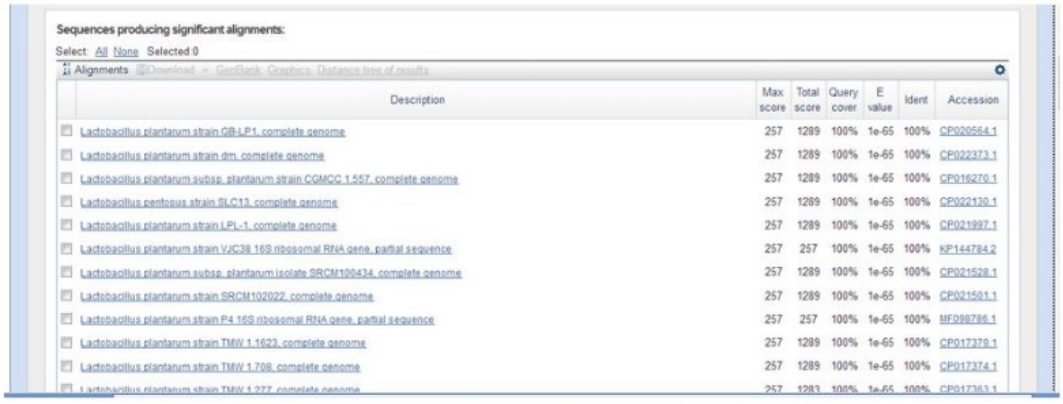
Presented in Figure 6 that the isolate is *Lactobacillus plantarum*

The following is Figure 7. containing the sequencing result of LAB No 3,

3. **Bac3**

**Consensus :**

GAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGAGAGTTTGTAACACCCAAA
GTCGGTGGGGTAACCTTTTAGGAACCAGCCGCCTAAGGTGGGACAGATGATTAGGGTGAA
GTCGTAACAAGGTAGCC



**Sequences producing significant alignments:**

Select: All None Selected:0

Alignments Download GenBank Graphics Distance tree of results

| Description | Max score | Total score | Query cover | E value | Ident | Accession |
|---|---|---|---|---|---|---|
| Lactobacillus plantarum strain GB-LP1, complete genome | 257 | 1289 | 100% | 1e-65 | 100% | CP020564.1 |
| Lactobacillus plantarum strain dm, complete genome | 257 | 1289 | 100% | 1e-65 | 100% | CP022373.1 |
| Lactobacillus plantarum subsp. plantarum strain CGMCC 1.557, complete genome | 257 | 1289 | 100% | 1e-65 | 100% | CP016270.1 |
| Lactobacillus pentosus strain SLC13, complete genome | 257 | 1289 | 100% | 1e-65 | 100% | CP022130.1 |
| Lactobacillus plantarum strain LPL-1, complete genome | 257 | 1289 | 100% | 1e-65 | 100% | CP021997.1 |
| Lactobacillus plantarum strain VJC38 16S ribosomal RNA gene, partial sequence | 257 | 257 | 100% | 1e-65 | 100% | KP144784.2 |
| Lactobacillus plantarum subsp. plantarum isolate SRCM100434, complete genome | 257 | 1289 | 100% | 1e-65 | 100% | CP021528.1 |
| Lactobacillus plantarum strain SRCM102022, complete genome | 257 | 1289 | 100% | 1e-65 | 100% | CP021501.1 |
| Lactobacillus plantarum strain P4 16S ribosomal RNA gene, partial sequence | 257 | 257 | 100% | 1e-65 | 100% | MF098786.1 |
| Lactobacillus plantarum strain TMW 1.1623, complete genome | 257 | 1289 | 100% | 1e-65 | 100% | CP017379.1 |
| Lactobacillus plantarum strain TMW 1.708, complete genome | 257 | 1289 | 100% | 1e-65 | 100% | CP017374.1 |
| Lactobacillus plantarum strain TMW 1.277, complete genome | 257 | 1283 | 100% | 1e-65 | 100% | CP017363.1 |

Figure 7. The isolate sequencing result on LAB No 3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Presented in Figure 7, it can be seen that LAB isolate of sample No 3 is *Lactobacillus plantarum*

The sequencing result of isolate sample No 4 is pointed out in Figure 8 below,

4. **Bac4**

**Consensus:**

CGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGAGAGTTTGTAACACC
CGAAGCCGGTGGCGTAACCCTTTTAGGGAGCGAGCCGTCTAAGGTGGGACAAATGATTAG
GGTGAAGTCGTAACAAGGTAGCCGTAA

Sequences producing significant alignments:

Select: All None Selected:0

Alignments Download GeoRank Graphics Distance tree of results

| Description | Max score | Total score | Query cover | E value | Ident | Accession |
|---|---|---|---|---|---|---|
| Bacterium strain A2 16S ribosomal RNA gene, partial sequence | 276 | 276 | 100% | 3e-71 | 100% | KX268350.1 |
| Lactobacillus paracasei strain HD1.7 16S ribosomal RNA gene, partial sequence | 276 | 276 | 100% | 3e-71 | 100% | EU249147.1 |
| Lactobacillus rhamnosus strain L156.4 16S ribosomal RNA gene, partial sequence | 274 | 274 | 99% | 1e-70 | 100% | KX644947.1 |
| Lactobacillus casei strain LC5, complete genome | 274 | 1372 | 99% | 1e-70 | 100% | CP017065.1 |
| Lactobacillus rhamnosus strain 4B15, complete genome | 274 | 1372 | 99% | 1e-70 | 100% | CP021426.1 |
| Lactobacillus paracasei strain 8A, complete genome | 274 | 1372 | 99% | 1e-70 | 100% | CP014985.1 |
| Lactobacillus rhamnosus strain Pen, complete genome | 274 | 1372 | 99% | 1e-70 | 100% | CP020464.1 |
| Lactobacillus rhamnosus strain WQ2 genome | 274 | 274 | 99% | 1e-70 | 100% | CP020016.1 |
| Lactobacillus rhamnosus strain BFE5264, complete genome | 274 | 1372 | 99% | 1e-70 | 100% | CP014201.1 |
| Lactobacillus casei strain K1C 16S ribosomal RNA gene, partial sequence; 16S-23S ribosomal RNA intergenic spacer, complete sequence; and 23S | 274 | 274 | 99% | 1e-70 | 100% | KU954559.1 |
| Lactobacillus rhamnosus strain LRB, complete genome | 274 | 1372 | 99% | 1e-70 | 100% | CP016823.1 |
| Lactobacillus rhamnosus gene for 16S ribosomal RNA, partial sequence, strain: NGRI 0110 | 274 | 274 | 99% | 1e-70 | 100% | LC177236.1 |
| Lactobacillus paracasei strain FS3 16S ribosomal RNA gene, partial sequence | 274 | 274 | 99% | 1e-70 | 100% | KU315064.1 |
| Lactobacillus rhamnosus strain ABCC 290 genome | 274 | 274 | 99% | 1e-70 | 100% | CP014645.1 |

Figure 8. The isolate sequencing result on LAB No 4

It is identified within Figure 8 that the isolate of sample No 4 is *Lactobacillus paracasei*

Then, molecular identification was performed with the second four isolates by conducting the analysis of these four isolates consecutively. For the bacteria DNA of PCR amplification result on 165 ribosomal sequencing zone is highlighted in the following Figure 9.,

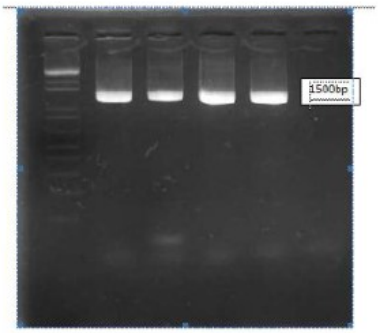


**Figure 9. Electrophoresis result on 4 isolates; M0. A5 , M16.16.2 and M16.4**


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Picture Description:

- The sample of PCR product is in the size of 1500bp, (-) control negative
- Ladder DNA 1kb plus 100, 200, 300, 400, 500, 650, 850, 1000, 1650, 2000, 3000, 4000 bp.
  - Fasta format was obtained from the result of sample sequencing analysis using Bioedit program. The fasta format of the sample is as follow:

>CONTIQ_M16.4_1440bp_*Lactobacillus plantarum*_100%

GCTCAGGACGAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAACGAACTCTGGTATTGATTGGTGCTT
GCATCATGATTTACATTTGAGTGAGTGGCGAACTGGTGAGTAACACGTGGGAAACCTGCCCAGAAGCGGGGGATA
ACACCTGGAAACAGATGCTAATACCGCATAACAACTTGGACCGCATGGTCCGAGCTTGAAAGATGGCTTCGGCTAT
CACTTTTGGATGGTCCCGCGGCGTATTAGCTAGATGGTGGGGTAACGGCTCACCATGGCAATGATACGTAGCCGAC
CTGAGAGGGTAATCGGCCACATTGGGACTGAGACACGGCCCAAACTCCTACGGGAGGCAGCAGTAGGGAATCTTC
CACAATGGACGAAAGTCTGATGGAGCAACGCCGCGTGAGTGAAGAAGGGTTTCGGCTCGTAAAACTCTGTTGTTA
AAGAAGAACATATCTGAGAGTAACTGTTCAGGTATTGACGGTATTTAACCAGAAAGCCACGGCTAACTACGTGCCA
GCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGATTTATTGGGCGTAAAGCGAGCGCAGGCGGTTTTTTA
AGTCTGATGTGAAAGCCTTCGGCTCAACCGAAGAAGTGCATCGGAAACTGGGAAACTTGAGTGCAGAAGAGGACA
GTGGAACTCCATGTGTAGCGGTGAAATGCGTAGATATATGGAAGAACACCAGTGGCGAAGGCGGCTGTCTGGTCT
GTAACTGACGCTGAGGCTCGAAAGTATGGGTAGCAAACAGGATTAGATACCCTGGTAGTCCATACCGTAAACGAT
GAATGCTAAGTGTTGGAGGGTTTCCGCCCTTGAGTGCTGCAGCTAACGCATTAAGCATTCCGCCTGGGGAGTACGG
CCGCAAGGCTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCTAC
GCGAAGAACCTTACCAGGTCTTGACATACTATGCAAATCTAAGAGATTAGACGTTCCCTTCGGGGACATGGATACA
GGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATTATC
AGTTGCCAGCATTAAGTTGGGCACTCTGGTGAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAA
TCATCATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGGATGGTACAACGAGTTGCGAACTCGCGAGAGTA
AGCTAATCTCTTAAAGCCATTCTCAGTTCGGATTGTAGGCTGCAACTCGCCTACATGAAGTCGGAATCGCTAGTAAT
CGCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGAGAGTTTGTAAC
ACCCAAAGTC

>CONTIQ_A5_1430bp_*Lactobacillus plantarum*_100%

ACGCTGGCGGCGTGCCTAATACATGCAAGTCGAACGAACTCTGGTATTGATTGGTGCTTGCATCATGATTT
ACATTTGAGTGAGTGGCGAACTGGTGAGTAACACGTGGGAAACCTGCCCAGAAGCGGGGGATAACACCTGGAAA
CAGATGCTAATACCGCATAACAACTTGGACCGCATGGTCCGAGCTTGAAAGATGGCTTCGGCTATCACTTTTGGATG
GTCCCGCGGCGTATTAGCTAGATGGTGGGGTAACGGCTCACCATGGCAATGATACGTAGCCGACCTGAGAGGGTA
ATCGGCCACATTGGGACTGAGACACGGCCCAAACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCACAATGGACG
AAAGTCTGATGGAGCAACGCCGCGTGAGTGAAGAAGGGTTTCGGCTCGTAAAACTCTGTTGTTAAAGAAGAACAT
ATCTGAGAGTAACTGTTCAGGTATTGACGGTATTTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGT
AATACGTAGGTGGCAAGCGTTGTCCGGATTTATTGGGCGTAAAGCGAGCGCAGGCGGTTTTTTAAGTCTGATGTGA
AAGCCTTCGGCTCAACCGAAGAAGTGCATCGGAAACTGGGAAACTTGAGTGCAGAAGAGGACAGTGGAACTCCAT
GTGTAGCGGTGAAATGCGTAGATATATGGAAGAACACCAGTGGCGAAGGCGGCTGTCTGGTCTGTAACTGACGCT
GAGGCTCGAAAGTATGGGTAGCAAACAGGATTAGATACCCTGGTAGTCCATACCGTAAACGATGAATGCTAAGTG
TTGGAGGGTTTCCGCCCTTCAGTGCTGCAGCTAACGCATTAAGCATTCCGCCTGGGGAGTACGGCCGCAAGGCTGA
AACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCTACGCGAAGAACCTT
ACCAGGTCTTGACATACTATGCAAATCTAAGAGATTAGACGTTCCCTTCGGGGACATGGATACAGGTGGTGCATGG
TTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATTATCAGTTGCCAGCATT
AAGTTGGGCACTCTGGTGAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCT
TATGACCTGGGCTACACACGTGCTACAATGGATGGTACAACGAGTTGCGAACTCGCGAGAGTAAGCTAATCTCTTA
AAGCCATTCTCAGTTCGGATTGTAGGCTGCAACTCGCCTACATGAAGTCGGAATCGCTAGTAATCGCGGATCAGCA
TGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGAGAGTTTGTAACACCCAAAGTCG


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>CONTIQ_M16.16.2_1422bp_*Lactobacillus plantarum*_99%
TGGTTCCTAAAAGGTTACCCCACCGACTTTGGGTGTTACAAACTCTCCATGGTGTGACGGGCGGTGTGTAC
AAGGCCCGGGAACGTATTCACCGCGGCATGCTGATCCGCGATTACTAGCGATTCCGACTTCATGTAGGCGAGTTGC
AGCCTACAATCCGAACTGAGAATGGCTTTAAGAGATTAGCTTACTCTCGCGAGTTCGCAACTCGTTGTACCATCCATT
GTAGCACGTGTGTAGCCCAGGTCATAAGGGGCATGATGATTTGACGTCATCCCCACCTTCCTCCGGTTTGTCACCGG
CAGTCTCACCAGAGTGCCCAACTTAATGCTGGCAACTGATAATAAGGGTTGCGCTCGTTGCGGGACTTAACCCAACA
TCTCACGACACGAGCTGACGACAACCATGCACCACCTGTATCCATGTCCCCGAAGGGAACGTCTAATCTCTTAGATT
GCATAGTATGTCAAGACCTGGTAAGGTTCTTCGCGTAGCTTCGAATTAAACCACATGCTCCACCGCTTGTGCGGGCC
CCCGTCAATTCCTTTGAGTTTCAGCCTTGCGGCCGTACTCCCCAGGCGGAATGCTTAATGCGTTAGCTGCAGCACTGA
AGGGCGGAAACCCTCCAACACTTAGCATTCATCGTTTACGGTATGGACTACCAGGGTATCTAATCCTGTTTGCTACCC
ATACTTTCGAGCCTCAGCGTCAGTTACAGACCAGACAGCCGCCTTCGCCACTGGTGTTCTTCCATATATCTACGCATT
TCACCGCTACACATGGAGTTCCACTGTCCTCTTCTGCACTCAAGTTTCCCAGTTTCCGATGCACTTCTTCGGTTGAGCC
GAAGGCTTTCACATCAGACTTAAAAAACCGCCTGCGCTCGCTTTACGCCCAATAAATCCGGACAACGCTTGCCACCT
ACGTATTACCGCGGCTGCTGGCACGTAGTTAGCCGTGGCTTTCTGGTTAAATACCGTCAATACCTGAACAGTTACTCT
CAGATATGTTCTTCTTTAACAACAGAGTTTTACGAGCCGAAACCCTTCTTCACTCACGCGGCGTTGCTCCATCAGACT
TTCGTCCATTGTGGAAGATTCCCTACTGCTGCCTCCCGTAGGAGTTTGGGCCGTGTCTCAGTCCCAATGTGGCCGATT
ACCCTCTCAGGTCGGCTACGTATCATTGCCATGGTGAGCCGTTACCCCACCATCTAGCTAATACGCCGCGGGACCAT
CCAAAAGTGATAGCCGAAGCCATCTTTCAAGCTCGGACCATGCGGTCCAAGTTGTTATGCGGTATTAGCATCTGTTT
CCAGGTGTTATCCCCCGCTTCTGGGCAGGTTTCCCACGTGTTACTCACCAGTTCGCCACTCACTCAAATGTAAATCAT
GATGCAAGCACCAATCAATACCAGAGTTCGTT

>CONTIQ_MO_797bp_*Lactobacillus plantarum*_100%
AACACTTAGCATTCATCGTTTACGGTATGGACTACCAGGGTATCTAATCCTGTTTGCTACCCATACTTTCGAGCCTCA
GCGTCAGTTACAGACCAGACAGCCGCCTTCGCCACTGGTGTTCTTCCATATATCTACGCATTTCACCGCTACACATGG
AGTTCCACTGTCCTCTTCTGCACTCAAGTTTCCCAGTTTCCGATGCACTTCTTCGGTTGAGCCGAAGGCTTTCACATCA
GACTTAAAAAACCGCCTGCGCTCGCTTTACGCCCAATAAATCCGGACAACGCTTGCCACCTACGTATTACCGCGGCT
GCTGGCACGTAGTTAGCCGTGGCTTTCTGGTTAAATACCGTCAATACCTGAACAGTTACTCTCAGATATGTTCTTCTT
TAACAACAGAGTTTTACGAGCCGAAACCCTTCTTCACTCACGCGGCGTTGCTCCATCAGACTTTCGTCCATTGTGGAA
GATTCCCTACTGCTGCCTCCCGTAGGAGTTTGGGCCGTGTCTCAGTCCCAATGTGGCCGATTACCCTCTCAGGTCGGC
TACGTATCATTGCCATGGTGAGCCGTTACCCCACCATCTAGCTAATACGCCGCGGGACCATCCAAAAGTGATAGCCG
AAGCCATCTTTCAAGCTCGGACCATGCGGTCCAAGTTGTTATGCGGTATTAGCATCTGTTTCCAGGTGTTATCCCCCG
CTTCTGGGCAGGTTTCCCACGTGTTACTCACCAGTTCGCCACTCACTCAAATGTAAATCATGATGCAAGCACCAATCA
ATACCAGAGTTCGTTCGA

The sequence molecular identification result of the second sample on DNA 4 is shown in Table 2. as follow:

**Table 2. Result of LAB molecular identification**

| No. | Isolate Code | LAB Type |
|---|---|---|
| 1. | **IsolatMO** | *Lactobacillus plantarum* |
| 2. | **IsolatM16.16.2**, | *Lactobacillus plantarum* |
| 3. | **IsolatA5** | *Lactobacillus plantarum* |
| 4. | **Isolat16.4**, | *Lactobacillus plantarum* |

**Phylogenetics Analysis**

In the following four samples, phylogenetics analysis was carried out using the **bootsrap method** as seen in Figure 10 below:


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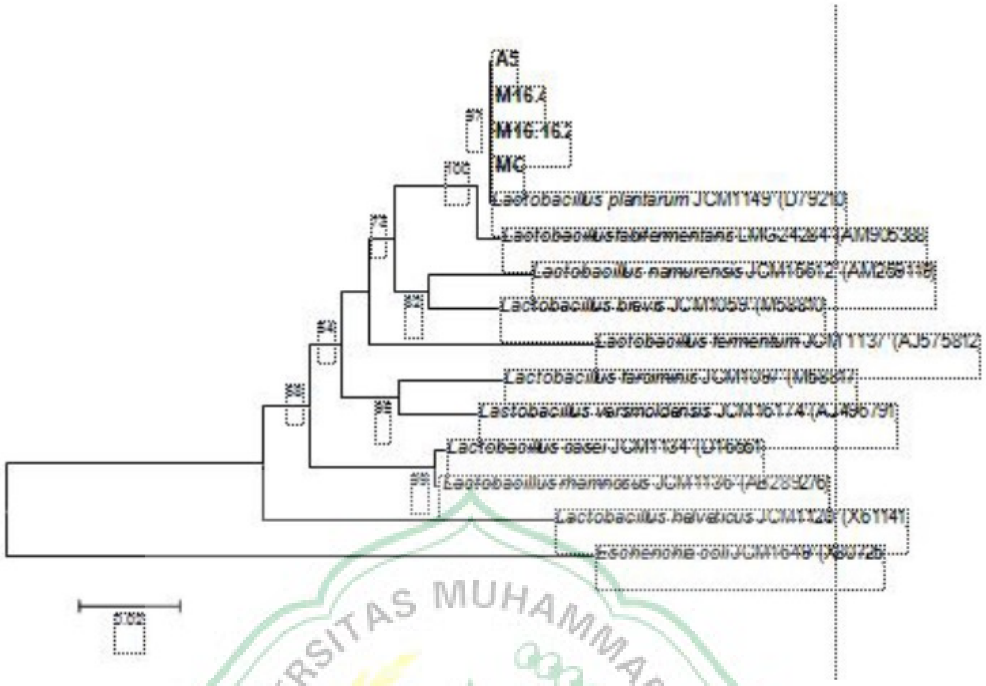


Figure 10. Phylogenetic tree obtained from Neighbor Joining analysis with 1000 repetition

## CONCLUSION

Regarding to the research conducted and the results obtained, it can be concluded that:

1. A total of 134 isolates of BAL (Lactic Acid Bacteria) can be isolated from the fermentation process of coconut milk consisting of 97 isolates deriving from the oil layer, 23 isolates deriving from Blondo and 14 isolates deriving from the water layer.
2. There are six Lactic Acid bacteria that can be identified morphologically, namely *Lactobacillus plantarum, Lactobacillus paracasei, Lactobacillus thermobacterium, Micrococcus luteus, Corineaebacterium bovis dan Corineaebacterium xerocis.*
3. The molecularly identified Lactic Acid Bacteria using PCR is broken down into three types, namely Lactobacillus plantarum, *Lactobacillus plantarum* strain JCM1149T, and *Lactobacillus paracasei.*

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