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# Probiotic research in several products of virgin coconut oil from Padang, Indonesia

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**Abstract.** Virgin Coconut Oil (VCO), have been produce by community people in Padang West Sumatra, Indonesia, with diffrent quality and the process. Most of the process using spontaneos fermentation methods with diffrent location of making and environment. We are interested in probiotic research in VCO samples to find good or new Lactic Acid Bacteria (LAB) as probiotic and antimicrobial to maintain good health. There were 7 VCO sampels have been taken from local market that have been isolated and characterized antimicrobial activity and molecular spesies. The were 11 LAB colonies have been found. The majority of LAB samples were good inhibition zone agains indicator bacterias such as, *Escherichia coli*, *Staphylococcus aureus* and *Bacillus subtilis*. Ampicillin Sodium 100 µg/mL was used as positive control. The results shows the inhibition zone was obtained from sample 1A (12 mm), agains *E.coli*, sample 6C (13.5 mm) for *Bacillus* and 3A (15 mm), for *S. aureus* as at low pH from 3 to 5 and high temperature from 60 to 100 degee Celcius. Ssequence analysis of 11 isolates are diversity of *Lactobacillus plantarum* species with different strains and new diversity of *Lactobacillus sakei*.

## 1. Introduction

Probiotic reserch have been useful to find good LAB as useful microorganism to use in our bodies and several applicatio n in animal feed, also enviroment. Virgin Coconut Oil (VCO) made from fermentation methods, normally rich in lauric acid contain, wich is antiviral, antibacterial and antiprotozoa and can cope viral attacks such as influenza and HIV [1, 2]. Other beneficial of VCO also contain LAB as antimicrobial [2, 3]. Lactic Acid Bacteria (BAL) can be used as a substitute for chemical preservatives in VCO, which aims to extend the shelf life of the product and has some positive effects on humans such as guarding of gastrointestinal infections, intolerant lactose reduction, reducing constipation, also anticarcinogenic effects [3, 4]. VCO also have been reported as anticholesterol, improve immune system, antiinflammation, and anti cancer [5, 6]. This is why we determined the important LAB in several VCO products from local market and classified as good supplement probiotics as most antiinflammation and antmicrobial [7, 8].The LAB has the ability to produce components antimicrobials through the production of organic acids, hydrogen peroxide, diacetyl, anti-fungal components such as lactic acid or phenillactic acid and bacteriocin. It is expected



Lactic Acid Bacteria (BAL) can be a natural chemical preservative that is able to maintain the natural safety and consumption of food products such as VCO [8, 9].

## 2. Material and methods

*2.1 Sample Collection, VCO sample which made in Padang areas, was collected randomly.*

### *2.2. Activity of LAB Against Indicator Bacteria from Virgin Coconut Oil (VCO)*

Start from serial dilution technique was used [9, 10]. MRS broth medium was used, incubated at 37°C for 24 hours in an aerobic condition. The plates were observed for appearance of colonies and number of colonies produced on plate. Bacteria were purified by streak plate method on MRS agar and incubated at 37°C for 48 hours and then maintained in refrigerator at 4°C till further analysis. All isolated were chosen and initially identified with the classical microbiological methods of gram stain, catalase reactions, fermentation type, and growth phase. Gram staining and microscopic were reported.

### *2.3. Activity of supernatant LAB Against Indicator Bacteria*

The method used for the antibacterial of BAL was well diffusion method. [9, 10]. Indicator bacteria have been cultured at 24 hours in Luria Bertani medium. Add and pipetted into a petri dish containing media Muller Hinton Agar. The  $\pm 6.5$  mm diameter hole was prepared. The plate was then incubated at 30 °C. for 24 hours, for testing the supernatant inhibitory ability of the test bacteria. The inhibitory zone formed is an inhibition area of the BAL antimicrobial substrate against the test bacteria. The inhibitory zone of clear areas appearing around the well is observed and measured with a sliding range. Each clear zone is measured in diameter four times in different places and the result is averaged.

### *2.4 Resistance Testing Against Acid and Heat Sensitivity of LAB*

Cell-free supernatant culture was suspected to contain antimicrobial bacteriocin, was heated for 10 min at 60 °C, 70 °C, 80 °C, 90 °C, 100 degree Celsius, and bacteriocin activity were tested by using to counteract the indicator bacteria using well diffusion methods. The sensitivity of bacteriocin to different pH was tested in supernatant cultures with pH 3.0, 4.5, 7.0 and 9.0 susceptibility [9, 10].

### *2.5 Isolation Genomic DNA and 16S Ribosomal (rRNA) gene amplification*

The 16S rRNA gene fragment of ~1.5 kb was amplified by using a pair of universal primers 27 F: (5'-AGAGTTTGTACCTGGCTAG- 3') and 1525 R: (5'-AGAAAGGAGGTGATCCAGCC-3'), [1, 2]. The DNA was analyzed by using 1.0% (w/v) agarose gel electrophoresis in 1x TAE buffer at 100 V for 30 min; and was visualized by using gel documentation system (Biodoc Analyze, Biometra). The purified PCR product was sequenced with 16S rRNA primers.

PCR Purification Products, Sequencing and Analysis: Purified PCR products was using the Fast Gen Gel / PCR Extraction Kit (Nippon Genetics, Germany). All fragment gene sequences are used to view proximity by using BLAST program in NCBI GenBank database which can be viewed on the website <http://blast.ncbi.nlm.nih.gov/Blast.cgi>. Sequences alignments are performed using ClustalW by <http://clustalW.ddbj.nig.ac.jp>. Phylogenetic trees are made using MEGA 7 applications.

### *2.6 Determination of Lactic Acid Concentration*

As much as 50 mL bacteria test were cultured during stationary phase, centrifuged at 13,000 rpm for 15 min at 4 °C, then filtered with 0.2µm porous filter paper. Pipetted 20 mL of the supernatant in a 100 mL glass and titrated with 0.5 N NaOH. Titrated until the pink color appears in the solution. Use the Phenolphthalein indicator (0.5% in 5% alcohol) as an indicator [10].

### 3. Results and discussion

The total colony of LAB vary among 7 VCO samples, around  $10^7$  to  $10^8$  CFU/mL, while not all VCO sample shown containing LAB.

**Tabel 1.** Total colony counting in 7 VCO samples

No.	Code Sample	Total Colony (CFU/mL)
1.	Sample 1	$1 \times 10^7$
2.	Sample 2	-
3.	Sample 3	$1 \times 10^7$
4.	Sample 4	-
5.	Sample 5	-
6.	Sample 6	$9.4 \times 10^8$
7.	Sample 7	$3.4 \times 10^8$

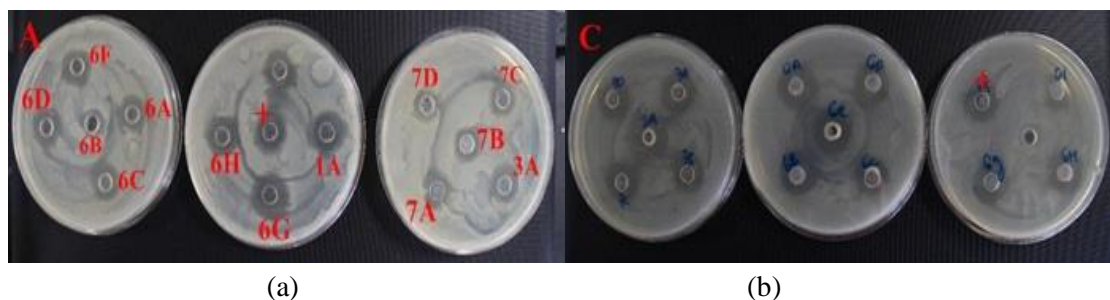
**Tabel 1**, explain the total colony counting of LAB, in 7 products of VCO, while sample no 1,3,6 and 7 showed good number of alive LAB for further determination. Not all VCO samples shows LAB, it probably different ways of making and environment [8, 9].

**Tabel 2.** Inhibition zone of LAB againsts bacterial Indicator such as *E.coli*, *B.subtilis* and *S.aureus*.

No.	Sample	<i>E. coli</i> (mm)	<i>B. Subtilis</i> (mm)	<i>S. aureus</i> (mm)
1	1A	$12 \pm 0.2887^a$	$10.25 \pm 0.4330^a$	$13.25 \pm 0.1443^a$
2	3A	$8.25 \pm 0.1443^a$	$9.25 \pm 0.4330^a$	$15 \pm 0.2887^a$
3	6A	$10.75 \pm 0.1443^a$	$11.25 \pm 0.1443^a$	$13.75 \pm 0.1443^a$
4	6C	$10 \pm 0.2887$	13.5	11.5
5	6D	$11 \pm 0.2887^a$	$11.25 \pm 0.1443^a$	13.5
6	6G	$12.25 \pm 0.4330^b$	$10 \pm 4.410^b$	$13.5 \pm 0.1667^b$
7	6H	$12 \pm 0.2887^{ac}$	$10.75 \pm 0.1443^a$	$11.5 \pm 0.667^c$
8	7A	11.5	$10.75 \pm 0.1443^c$	$11.5 \pm 0.6009^c$
9	7B	$8.75 \pm 0.1443^a$	$9.25 \pm 0.1443^a$	$11.25 \pm 0.4330^a$
10	7C	$8.75 \pm 0.1443^a$	$8.75 \pm 0.1443^a$	$11.75 \pm 0.1443^a$
11	7D	$8.25 \pm 0.1443^a$	$8.75 \pm 0.1443^a$	$13 \pm 0.2887^a$

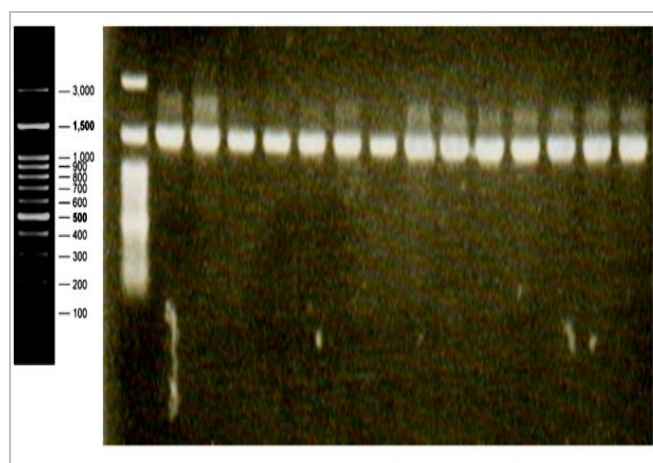
Average of 3 times means ,a (p.v < 0.01), b (p.v < 0.05), c (p.v > 0.05)

Sample with code 3A, is the highest holozone againsts three bacterial indicator, and continue to study the phylogenetic species. Antimicrobial properties are generated due to influence of acids on bacterial cytoplasm membranes that affect the active transport and membrane potential. [10, 11]. The holozone can be seen in **figure 1**, below.



**Figure 1.** Inhibition zone of 7 VCO sample againsts (a) *E. coli* and (b) *S. aureus*

Isolation Genomic DNA and 16S Ribosomal (rRNA) gene amplification with PCR DNA was used for the amplification of the 16S rRNA gene using PCR (Polymerase Chain Reaction) technique. The resulting amplification product was 1500 bp (**Figure 2**)



**Figure 2.** Results of 11 colonies from 7 VCO samples of LAB, using 16S rRNA gene Amplification with PCR

### 3.1 Nucleotide Sequence Analysis

These bases are analyzed with NCBI data through BLAST (Basic Local Alignment Search Tool) program [13, 14]. Based on the phylogenetic tree, it is known that 11 isolates are bacteria with *Lactobacillus plantarum* species with different strains and new diversity of *Lactobacillus sakei* shown in Table 3.

All 11 colonies of LAB isolates from different samples of Virgin Coconut Oil, see Table 4, conformed as good VCO and contain diversity of *Lactobacillus plantarum*, and new *Lactobacillus sakei*. The strongest antimicrobial pathogen bacteria can be used as probiotic performed acids pH and high temperature resistant [14, 15].

**Table 3.** Sequens of new LAB from 11 colonies in 7 VCO samples

No.	Sample Code	LAB Isolated
1.	1A	<i>Lactobacillus plantarum</i> strain PON100536
2.	3A	<i>Lactobacillus plantarum</i> strain C410L1
3.	6A	<i>Lactobacillus plantarum</i> strain DS2 KCTC12992BP
4.	6C	<i>Lactobacillus plantarum</i> strain L41
5.	6D	<i>Lactobacillus sakei</i> WIKIM49
6.	6G	<i>Lactobacillus sakei</i> strain SH15
7.	6H	<i>Lactobacillus sakei</i> strain PR11
8.	7A	<i>Lactobacillus sakei</i> strain KKD
9.	7B	<i>Lactobacillus plantarum</i> strain MF1298
10.	7C	<i>Lactobacillus plantarum</i> strain LY-78
11.	7D	<i>Lactobacillus plantarum</i> strain NM22-22

### 3.2 Lactic Acid Concentration

In Tabel 4, explain the oncentration of Lactic Acid, for *lactobasillus plantarum*, ranges from 0.2 to 0.3 M, this is also range for good antimicrobial and anti fungi [15, 16].

**Table 4.** Lactic Acid Concentrations of Lactic Acid Bacteria (BAL)

No.	Sample Code	Species	mL NaOH	Lactic Acid Concentration (M)
1.	1A	<i>Lactobacillus plantarum</i> strain PON100536	5.5	0.2725
2.	3A	<i>Lactobacillus plantarum</i> strain C410L1	5.6	0,2775
3.	6A	<i>Lactobacillus plantarum</i> strain DS2 KCTC12992BP	5.8	0.2950
4.	6C	<i>Lactobacillus plantarum</i> strain L41	6	0.3050
5.	7B	<i>Lactobacillus plantarum</i> strain MF1298	6.3	0.31
6.	7C	<i>Lactobacillus plantarum</i> strain LY-78	5.7	0.2925
7.	7D	<i>Lactobacillus plantarum</i> strain NM22-22	6.2	0.3050

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