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ACCEPTANCE LETTER

Padang, 08th September 2017

Our ref: 125/LoA/ICEE2017

Dear Horas Rajagukguk, Sumaryati Syukur, Syafrizayanti, Yolani Syaputri, Endang Purwati, Hitoshi Iwahashi

Greetings from 4th International Conference on Earth Sciences and Engineering (4th ICEE 2017)

First of all, thank you for your interest and research contribution to 4th International Conference on Earth Sciences and Engineering (4th ICEE-2017), during 29th-31st August, 2017 at Convention Hall, Andalas University, Padang, West Sumatra, Indonesia.

We are happy to inform you that based on the review process your paper will be published in a Scopus Indexed Journal.

Paper ID: **ICEE2017-125**
Title: STRONG ANTIMICROBIAL OF LACTIC ACID BACTERIA AND SPECIES IDENTIFICATION OF VIRGIN COCONUT OIL PRODUCTS IN PADANG WEST SUMATERA, INDONESIA
Author: Horas Rajagukguk, Sumaryati Syukur, Syafrizayanti, Yolani Syaputri, Endang Purwati, Hitoshi Iwahashi

The presented paper has been accepted and under process for publication in, **International Journal on Advance Science, Engineering and Information Technology (IJASEIT), indexed by Scopus**.

Thank you and good luck.

Yours sincerely,

Dr. Abdul Hakam
Conference Chair - 4th ICEE 2017
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STRONG ANTIMICROBIAL OF LACTIC ACID BACTERIA AND SPECIES IDENTIFICATION OF VIRGIN COCONUT OIL PRODUCTS IN PADANG WEST SUMATERA, INDONESIA

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Abstract **-Virgin Coconut Oil (VCO) products are available in the market of Padang west Sumatra Indonesia, Produced by lokal Home Industry. Coconut, *Cocos nucifera,* is an important member of the family *Arecaceae*, have been traditional used for food and medicine in peoples of West Sumatra. Differently with coconut itself, the oil of coconut , VCO not all accepted to use as food supplement because of the image of the oil will increase blood cholesterol. Recently several reports proved that VCO contain Medium Chain Fatty Acids, as Lauric Acids is metabolize quickly to produce high energy and has antimicrobial activities. Lactic Acids Bacteria, known as antimicrobial also, have been isolated from 7 VCO samples, and 4 samples have strong lactic acid bacteria as antimicrobial. All of isolates VCO in MRS medium are gram positive, catalase negative and homofermentative. All isolates are able to inhibit pathogen bacteria such as *Escherichia coli, Bacillus subtilis* and *Staphylococcus aureus*. Antimicrobial test was obtained, the largest inhibition zones from pathogen bacteria are by sample codes 1A (12 mm), 6C (13.5 mm), and 3A (15 mm), respectively. Ampicillin Sodium 100 µg/mL is used as a positive control. All isolates can inhibit pathogenic bacteria in pH range of 3-9, but gave negative results on pH 3 against *S. aureus.* All of Lactic Acid Bacteria was obtained to sequence analysis using 16 S rRNA showed that *Lactobacillus plantarum* strain PON100536, *Lactobacillus plantarum* strain C410L1, *Lactobacillus plantarum* strain MF1298, *Lactobacillus plantarum* strain LY-78, *Lactobacillus plantarum* strain NM22-22, *Lactobacillus plantarum* strain DS2 KCTC12992BP, and *Lactobacillus plantarum* strain L41 with concentration Lactic acid 0.2725, 0.2775, 0.2950, 0.3050, 0.31, 0.2925, 0.3050 M, respectively.**

Keywords : Virgin Coconut Oil; probiotic; antimicrobial activity, 16S rRNA; L. plantarum

I. Introduction

Virgin Coconut Oil (VCO) contains ± 53% lauric acid and ±7% caprilic acid which has C_{10} chain. Lauric acid absorbed by body will be converted into monolaurin and caprilic acid will be converted into monocaprin. Monolaurin is monoglyceride compound that is antiviral, antibacterial and antiprotozoa and can cope viral attacks such as influenza and HIV. Monocaprin in human body is beneficial for health to overcome sexual diseases[1]-[2]. To make Virgin Coconut Oil (VCO) can be done through 3 ways; mechanically, provocatively (with inducement) and enzymatically. The most widely used production process is enzymatic (fermentation) process without heating and adding harmful chemicals to make virgin coconut oil has better quality [3]. The enzymatic process to make and provocation is done naturally by the aid of lactic acid bacteria.

Lactic Acid Bacteria (LAB) are a group of Gram-positive bacteria, do not form spores, coccus or bacil and produce lactic acid as the main product during carbohydrate fermentation [3]-[4]. Growth and activity of LAB also have inhibitory effects on decomposition bacterial and pathogenic bacteria, such as *Salmonella enteritidis, Escherichia coli, and Staphylococcus aureus*[5]-[6].

One of the important attributes of a bacterium classified as probiotics is the ability to produce antimicrobial components through the production of organic acids, hydrogen peroxide, diasetil, anti-fungal components such as lactic acid or phenillactic acid and bacteriocin[7]-[8]. Lactic Acid Bacteria (BAL) can be a natural chemical preservative that able to maintain safety and consumption food. LAB creates an acidic environment by utilizing sugars and carbohydrates and forming lactic acid in a condition that is not favored by other microorganisms with lowering the pH or producing antimicrobial agents such as bacteriocin, which can inhibit the growth of other bacteria[9]-[10]

II. Material and Method

A. Sample Collection

An advanced commercial VCO sample which made in west Sumatra was collected randomly in Padang city.

B. Isolation and characterization of lactic acid bacteria from Virgin Coconut Oil (VCO)

For isolation of LAB, serial dilution technique was used[6]-[7]. One mL of sample was dissolved into 9 mL of MRS broth. After dissolving, they were shaken homogeneously and were incubated at 37°C for 24 hours in an aerobic condition. Serial dilution of 10^{-2} until 10^{-8} were made by pipetting 0,1 ml of previous dilution into 0,9 ml of MRS Broth. 0,1 ml of final dilution was inoculated to MRS agar plates and incubated at 37°C for 48 hours for bacterial growth. 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 40°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 followed methods by[7].

1) Activity of LAB Against Indicator Bacteria: The method used for activity of LAB test is well diffusion method [9]-[10]. A total of 100 µL test bacteria that have been cultured 24 hours in Luria Bertani pippeted into petri dish containing media Muller Hinton Agar. diameter holes (± 6.5 mm) were prepared using a corkeborer, 100 µL of each LAB were pipetted into each hole, stored in the refrigerator (temperature 4-7 ° C) for 1 hour to allow LAB seep into Agar, then incubated at 30 ° C for 24 hours. The indicator bacteria used were *Escherichia coli, Staphylococcus aureus* and *Bacillus subtilis*. The inhibit zone is measured by a sliding range. Each clear zone is measured and experiment was performed twice.

2) Effect pH and Heat Sensitivity of Antimicrobial Activity: An optimization study on the physical-chemical characterization of LAB samples [11]-[12]. Heat sensitivity assay, cell-free supernatant culture suspected was heated for 10 min at 60 ° C, 70 ° C, 80 ° C, 90°C, and 100° C, activities were tested by using well diffusion method with *Escherichia coli, Bacillus subtilis* and *Staphylococcus aureus JCM20624*. The sensitivity toward pH was tested with pH 3.0, 4.5, 7.0 and 9.0 velocity with addition of 5 N of hydrochloric acid (HCl) and or 5N sodium hydroxide (NaOH) and detected by diffusion of the wells into same bacteria.

3) Isolation Genomic DNA and 16S Ribosomal (rRNA) gene amplification: Isolates LAB were incubated for 24 hours into MRS broth and extracted using Extrap Soil DNA Kit Plus Ver.2. The 16S rRNA gene fragment of ~1.5 kb was amplified by using a pair of universal primers 27 F : (5'-AGAGTTTGATCCTGGCTAG-3') and 1525 R: (5'-AGAAAGGAGGTGATCCAGCC-3'), [13]-[14]. The amplifications were performed with initial denaturation at 95°C for 5 min, and with 25 cycles of denaturation at 94°C for 1 min; annealing at 56°C for 1 min and extension at 72°C for 1,5 m . 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.

4) 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.

5) Determination of Lactic Acid Concentration: 50 mL LAB was centrifuged at 13,000 rpm for 15 min at 4 °C,

filtered with 0.2µm porous filtration paper. Pipetted 20 mL of supernatant into 100 mL glass. Titrated with 0.5 N NaOH until the pink color appears in the solution. Use the Phenolphthalein indicator (0.5% in 5% alcohol) as an indicator.

III. Result and Discussion

A. Total Colonies of Lactic Acid Bacteria (BAL)

The media used in BAL isolation are MRS Agar and MRS Broth.

TABLE 1
Total Colonies of Lactic Acid Bacteria (LAB)

No.	Sample Code	Total Coloni (CFU/mL)
1.	Sample 1	1×10^7
2.	Sample 2	-
3.	Sample 3	1×10^7
4.	Sample 4	-
5.	Sample 5	-
6.	Sample 6	9.4×10^8
7	Sample 7	3.4×10^8

The macroscopic colony of BAL showed white small rounded colonies, shiny, slippery and convex edges.

B. Identification of Lactic Acid Bacteria (BAL) in Morphology

7 isolates obtained LAB were bacil and gram positive. The catalase test for all isolates showed negative results and all isolates were homofermentative.

TABLE 2
GRAM STAINING RESULTS, CATALASE TEST AND TYPE OF LAB FERMENTATION

Isolate LAB	Biochemical Characteristics		
	Gram Staining	Catalase Test	Tipe fermentasi
1A	Positive	Negative	Homofermentative
3A	Positive	Negative	Homofermentative
7B	Positive	Negative	Homofermentative
7C	Positive	Negative	Homofermentative
7D	Positive	Negative	Homofermentative
6A	Positive	Negative	Homofermentative
6C	Positive	Negative	Homofermentative

There are two type of lactic acid bacteria fermentation, homofermentative and heterofermentative. Homofermentative lactic acid bacteria only produced lactic acid as the main product of fermentation. Whereas heterofermentative lactic acid bacteria produced lactic acid, ethanol, other acids such as acetic acid and CO_2. [13]-[14].

C. Growth Phases of Lactic Acid Bacteria (LAB)

From the result of growth curve measurements using the spectrophotometer wavelength 600 nm, the following data are obtained:

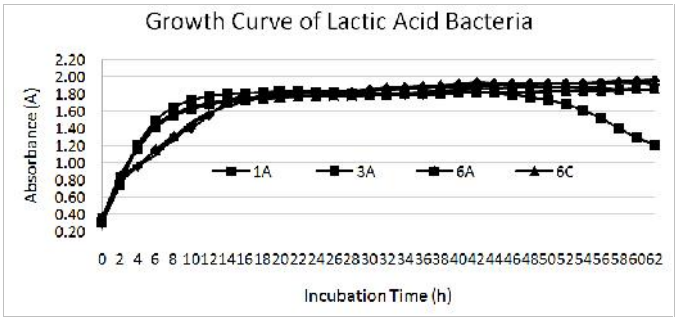

Fig 1 Growth Curve of 7 LAB Isolates in MRS broth Media

Fig 1 showed that all isolates do not have adaptation phase and at 0 to 14 hours of incubation time are exponential phases. All isolates stared the stationary phase from the 16 to 62 hours of incubation time, except samples with code 3A. Samples with code 3A stared death phase at 50 hours of incubation time. Stationary phase describes the accumulation of metabolites product from cell metabolism activities and nutrient content begins to run out, so that there is competition of nutrients and some cells die and others continue to grow. This causes the number of cells to be relatively constant. In this phase LAB began to produce secondary metabolites and lactic acid very slowly[15]-[16].

D. Activity of Isolate LAB Inhibit Indicator Bacteria

The test was performed with aim of looking at the antimicrobial activity of the isolate LAB obtained in inhibiting the growth of pathogenic bacteria. In this test the pathogen bacteria used were *Escherichia coli*, *Staphylococcus aureus* and *Bacillus subtilis.*

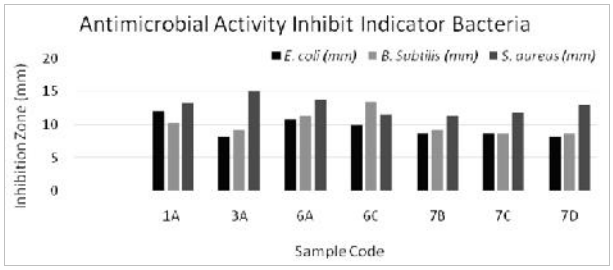

Fig 2 LAB Activity Inhibits Indicator Bacteria; *E.coli, B.subtilis* and *S.aureus*

Antibacterial test obtained in Fig 2, the majority of bacterial samples were good inhibit *S. aureus*. Ampicillin Sodium 100 µg/mL was used as positive control, showed clear zone is 14.25 mm. In the antimicrobial test with *E. coli* indicator bacteria, the largest inhibition zone was obtained from bacteria with sample code 1A (12 mm), while the smallest inhibition zone was obtained from bacteria with sample code 3A and 7D (8.25 mm). In the antimicrobial assay with *Bacillus subtilis*as indicator bacteria, the largest inhibition zone was obtained from bacteria with sample code 6C (13.5 mm), while the smallest inhibition zone was obtained from bacteria with sample code 7C and 7D (8.25 mm). In the antimicrobial assay with *S. aureus*as indicator bacteria, the largest inhibition zone was obtained from bacteria with sample

code 3A (15 mm), while the smallest inhibition zone was obtained from bacteria with sample code 7B (11.25 mm).

Antimicrobial mechanisms of BAL include the production of organic acids, hydrogen peroxide, diacetyl, and broad-spectrum antimicrobial compounds such as reuterin and bacteriocin. The antimicrobial effects are directly given by the presence of organic acids including lactate, acetate, and propionate. Antimicrobial properties are generated due to influence of acids on bacterial cytoplasmic membranes that affect the active transport and membrane potential. [17]-[18].

During growth, most of the sugar is converted by LAB into lactic acid which provides inhibitory power to other microorganisms. When lactic acid is produced, there is pH decreasing, which showed in unorganized or undissociated organic acids (SFAs). These undissociated acids are the main antimicrobial properties of LAB. These undissociated acids target and attack the bacterial membrane cause weakly acid anions to collect in the cytoplasm which further affects the metabolic process. There is growth inhibition and slowly die [15].

E. Isolate LAB Activity Toward Acid Resistance

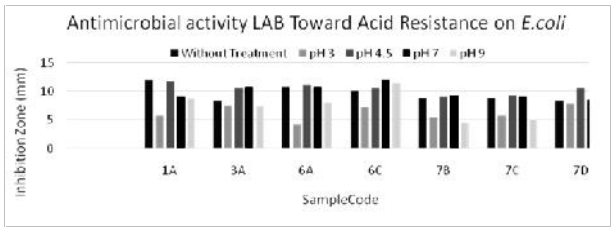

Fig 3 Antimicrobial activity LAB Toward Acid Resistance on *E.coli*

Fig 3 showed that acid resistance isolate LAB test on *E. coli* as indicator bacteria, it was found that good pH inhibit *E. coli* is range4.5 to 7. From the data, obtained isolate with code 1A has the best value inhibiting *E. Coli* at without treatment and pH 4.5 with clear zone 12mm. Isolate with code 7D has the best value inhibiting *E.coli* at pH 3 (7.75 mm). While isolate with code 6C has the best value inhibiting *E.coli* at pH 7 and 9 (12 and 11.5 mm, respectively). At pH 3 and 9, it still provides resistance to *E.coli*, but not as good as at pH 4.5 to 7. The sample code 6A has the smallest resistor at pH 3 (4.25 mm). The sample code 7B has the smallest resistor at pH 4.5 (9 mm). The 7D sample code has the smallest resistor at pH 7 (8.5 mm) and the sample codes 7B and 7D have the smallest resistor at pH 9 (4.5 mm).

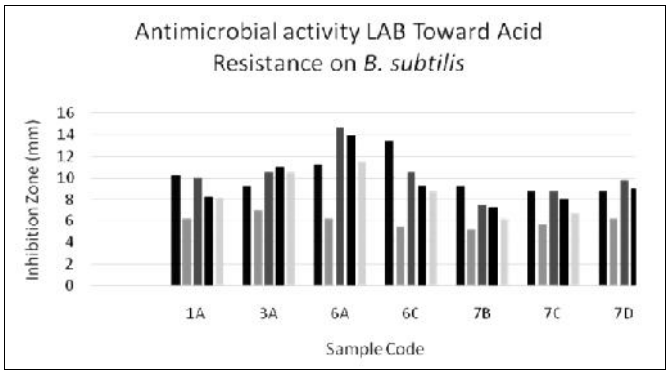

Fig 4 Antimicrobial activity LAB Toward Acid Resistance on *B. subtilis*

Antimicrobial activity LAB toward Acid Resistance on *B. subtilis* and ampicillin Sodium concentration was 100 µg/mL is used as positive control, and obtained clear zone is 14.25 mm. From the Fig 4 was found that lactic acid bacteria inhibited *Bacillus subtilis* with range pH 4.5 to 7. Sample with code 6C has the best value to inhibit indicator bacteria *Bacillus subtilis* without treatment (13.5 mm). Sample with code 3A has the best value to inhibit *Bacillus subtilis* at pH 3 (7 mm). Sample with code 6A has the best value inhibiting *Bacillus subtilis* at pH 4.5, 7 and 9 (14.75, 14 and 11.5 mm, respectively). At pH 3 and 9, lactic acid bacteria still provide inhibitory to indicator bacterial *Bacillus subtillis*, but not as good as at pH 4.5 to 7. The smallest inhibitory without treatment obtained from the sample with code 7C and 7D (8.75 mm) and the sample with the code 7B gave the smallest resistor value at pH 3, 4.5, 7 and 9 (5.25, 7.5, 7.25 and 6.25 mm, respectively).

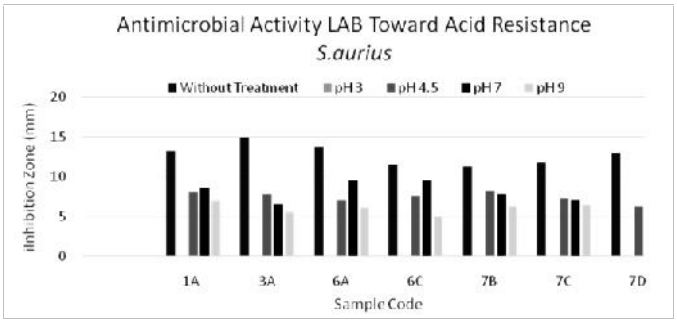

Fig 5 Antimicrobial activity LAB Toward Acid Resistance on *S.aurius*

Antimicrobial activity of lactic acid bacteria toward acid resistance on *S. aureus* found that lactic acid bacteria were able to inhibit bacteria at pH 4.5 to 9, but gave better results without treatment, the best inhibitory zone obtained from 3A (15 mm). At pH 3, lactic acid bacteria do not provide inhibitory zone. At pH 4.5, the best antimicrobial activity is showed by isolate with code 7B (8.25 mm). At pH 7, the best antimicrobial activity is showed by isolates with codes 6A and 6C with (9.5 mm). While At pH 9, the best antimicrobial activity is showed by isolate with code 1A (7 mm). While the smallest inhibition zone at pH 4.5 is present in a sample with code7D (6.25 mm) and at pH 7 the smallest inhibition zone is present in a sample with code 3A (6.5 mm) and at pH 9 the

smallest inhibitory zone is present in samples code 3A and 7D (5.5 mm).

LAB homoferrmentative produces other acids such as acetic acid and formic acid, under absence of glucose condition, and the presence of other carbohydrates for fermentation[16]- [17]. But none of these acids have a stronger antimicrobial effect than lactic acid. In addition some other small antimicrobial metabolites are also produced such as alcohol and aldehydes. In heterofermentative LAB there are fewer amounts of lactic acid produced when with acetic acid).

F. Isolate LAB Activity to Thermostability

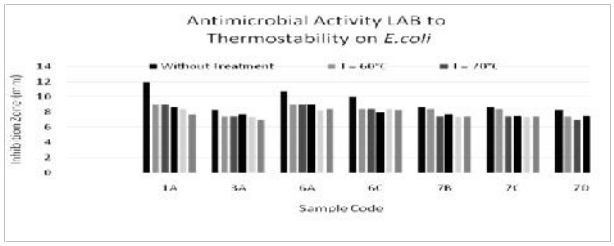

Fig 6 BAL Antimicrobial Activity Against Heat Resistance in *E.coli*

Antimicrobial activity of lactic acid bacteria to thermostability on *E.coli* was found that lactic acid bacteria were resistant to heat, where the results showed no significant difference inhibit *E.coli*. The Stability in high temperatur also found in thermophiles bacteria [15].

G. Isolation Genomic DNA and 16S Ribosomal (rRNA) gene amplification with PCR

Genetic diversity using 16S Ribosomal rRNA, have been followed[17]-18]. Extap Soil DNA kit Plus Ver.2 was used to isolate DNA that was previously LAB was overnight cultured in MRS Broth. Further isolated DNA was used for the amplification of the 16S rRNA gene using PCR (Polymerase Chain Reaction) technique. The amplification process used universal primer. The resulting amplification showed product was 1500 bp (Fig 7) with the appearance of clear and thick fragments in PCR products with equivalent to 1500 bp on DNA markers.

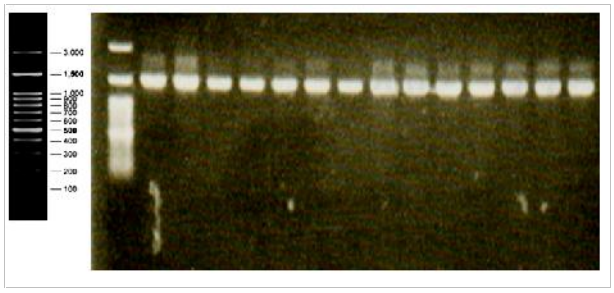

Fig 7 Results of 16S rRNA gene Amplification with PCR

PCR utilizes DNA polymerase enzymes that naturally play a role in DNA replication on replication process [17]- 18]. From the results of electrophoresis can be seen that the PCR process is good enough, where the DNA has a clear and bright band intensity without smears. The gel concentration used was 1% with 100 V for 21 min on the electrophoresis device. The samples were duplo.

H. Nucleotide Sequence Analysis

Before sequencing, PCR product was purified using the Fast Gen Gel/PCR Extraction Kit (Nippon Genetics, Germany). From the results performed in both directions, reverse and forward using universal primer. These bases is analyzed with NCBI data through BLAST (Basic Local Alignment Search Tool) program. The results showed the proximity of isolate species to several known species of their nucleotide sequence. Several species of bacteria with the closest identification values were analyzed by aligning sequence using the ClustalW online program (http://clustalW.ddbj.nig.ac.jp). The phylogenetic tree is formed in order to determine the relationship of species based on their similarities and genetic differences with MEGA7 applications [17]- [18] .

Based on the phylogenetic tree, it is known that 7 isolates are bacteria with *Lactobacillus plantarum*species with different strains.

TABLE 3.

ALL ISOLATES OF LACTIC ACID BACTERIAL SEQUENCES

No.	Sample Code	LAB Isolated	Accession Number
1.	1A	*Lactobacillus plantarum strain PON100536*	KJ921836.1
2.	3A	*Lactobacillus plantarum strain C410L1*	CP017954.1
3.	7B	*Lactobacillus plantarum strain MF1298*	CP013149.1
4.	7C	*Lactobacillus plantarum strain LY-78*	CP015308.1
5.	7D	*Lactobacillus plantarum strain NM22-22*	HM218107.1
6.	6A	*Lactobacillus plantarum strain DS2 KCTC12992BP*	KX470811.1
7.	6C	*Lactobacillus plantarum strain L41*	KP317710.1

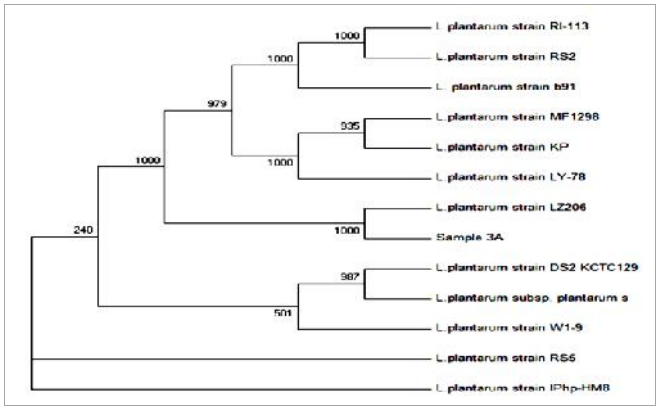

Fig 8 Phylogenetic Tree of Lactobacillus plantarum in 7 VCO samples

IV. Conclusion

All 7 LAB isolates from different sample of Virgin Coconut Oil, conformed as diversity of *plantarum* , and strong antimicrobial pathogen bacteria from pH range 3 to 9.

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