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Isolation and Molecular Characterization of Lactic Acid Bacteria by Using 16s rRNA from Fermented Buffalo Milk (Dadih) in Sijunjung, West Sumatera.

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ABSTRACT

Dadih is traditional fermented buffalo milk originated from West Sumatera. It contains some lactic acid bacteria (LAB) which has benefit for lowering cholesterol level in blood and has anticancer activities. LAB was isolated in MRS medium, then isolates were then characterized biochemically by Gram staining and catalase test. Total colony was 308 x 10^8 cfu/mL, Gram positive, and catalase negative. Six isolates randomly picked and their growth was measured by spectrophotometer. LAB isolates was then selected by their antimicrobial activity and acid tolerance (pH 3-4). Selected LAB isolates were R2, R3, and R5 showing good antimicrobial activity (± 12 mm against *E. coli,* ± 13.5 against *S. aureus*, and ± 12 mm against *S. typhi*) and having tolerance to acid. Three selected isolates were then identified by DNA molecular identification and amplified by using PCR with primer 27F (3' AGAGTTTGATCCTGGCTAG 5') and 1525 R (3' AGAAAGGAGGTGATCCAGCC 5') to determine the species of bacteria. From PCR product, only 2 isolates (R2 and R3) can be continued to sequence analysis. Phylogenetic tree showed that isolates R2 has better result and close relative to *L. plantarum* with 98% identification value.

Keywords: dadih, probiotic, antimicrobial activity, 16s rRNA, *L. plantarum*

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INTRODUCTION

Dadih, traditional fermented milk from West Sumatera, Indonesia is made by pouring fresh raw unheated buffalo milk into a bamboo tube capped with banana leaves, and then allowed to ferment for two days at room temperature. The milk is fermented by indigenous lactic acid bacteria of buffalo milk [1]. Buffalo milk is composed of total solid (16.39-18.47%), fat (6.57-7.97%), and crude protein (4.59-5.37%), while gross compositions of buffalo milk yogurt (e.g dadih) are total solid (11.60%), fat (0.68%), and protein (4.49%). Buffalo milk yogurt showed higher content of protein, total solid than reported for cow milk yogurt [2].

Lactic acid bacteria (LAB) contained in fermented milk can break down lactose. In addition, the rich content of lactic acid bacteria make fermented milk sugar levels to be much lower than fresh milk lactose content. The process of fermentation by LAB, 30-40% will break down the lactose into glucose and galactose that is easily absorbed by the body. *Lactobacillus brevis* isolated from green cacao fermentation in show lactase activities, which is very important enzyme for lactose intolerant people [3].

Many lactic acid bacteria (LAB) strains have been classified as probiotics due to their beneficial effects towards human health; in particular, the gastrointestinal (GI) tract. Those reported include prevention of colon cancer, reduction of cholesterol levels, inhibition of pathogenic microflora, stimulation of immune response, reduction of constipation, enhancement of lactose digestion in lactase deficient subjects as well as alleviation of food allergy [4-7]. Some studies on probiotic properties of indigenous lactic acid bacteria isolated from dadih have shown to exhibit antimutagenic, hypocholesterolemic, acid and bile tolerance as well as antipathogenic properties [8-11].

The aim of this work was to isolate lactic acid bacteria as probiotic and to determine the phylogenetic species isolated from fermented buffalo milk (dadih) which has antimicrobial activity.

MATERIALS AND METHODS

Isolation and characterization of lactic acid bacteria from dadih

Dadih samples were obtained from Pematang Panjang, Sijunjung, West Sumatera, Indonesia. For isolation of LAB, serial dilution technique was used. One gram 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 peptone water. 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. Six colonies are randomly chosen. All isolated were initially identified with the classical microbiological methods of Gram stain, catalase reactions, and growth phase.

pH tolerance

LAB isolates are grown on MRS broth then incubated for 24 hours at 37ºC. 1 mL of the culture was inoculated into 9 ml of MRS broth which has been set up pH of 3 and 4. Observe bacterial growth by using a spectrophotometer at 600 nm at 0, 2, 4, 12 and 24 hours.

Antagonist test using disc diffusion method

The antagonistic activity of the isolated LAB against pathogenic bacteria was determined by using agar disc diffusion method. Each of LAB isolate was propagated in MRS broth medium and incubated anaerobically at 37°C for 24 h. Filter paper disc (diameter of 6 mm, Whatman No.1) was immersed in culture for 1 m and air dried for 15 s. The paper discs were put on the agar surface pre-spread with indicator organism. Three indicator strains: *Eschericia coli*, *Staphylococcus aureus* and *Salmonella typhi* obtained from Medical Faculty (Andalas University) were used. The test was performed in triplicates. Then, the disc on the plates were incubated at 37°C for 24 h and the zone of inhibitions formed surrounding the disc were observed.

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16S Ribosomal (rRNA) gene amplification, sequencing and analysis

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'). The amplifications were performed with initial denaturation at 95°C for 5 min, and with 35 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 (Personal Termocycler, Biometra). The DNA was analyzed by using 1.0% (w/v) agarose gel electrophoresis (Mupid-Exu Submarine Electrophoresis System, Advance) 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. The sequences of the whole gene fragment were used for the similarity search against NCBI GenBank database using the BLAST program, available at website *http://blast.ncbi.nlm.nih.gov/Blast.cgi*.

RESULT AND DISCUSSION

Isolation and characterization of lactic acid bacteria from dadih

Total colony was 308 x 10^8 cfu/mL. Six colonies were picked as isolates were assigned as lactic acid bacteria based on the morphology, gram stained, and catalase test. They were observed Gram positive rod and catalase negative. All isolates showed the exponential growth phase between 4-18th hours and stationary phase start from 18th hour (Fig. 1). The results demonstrated the same growth curve of *L. plantarum* isolated from lump sheep's cheeses under anaerobic conditions at 37°C [12].

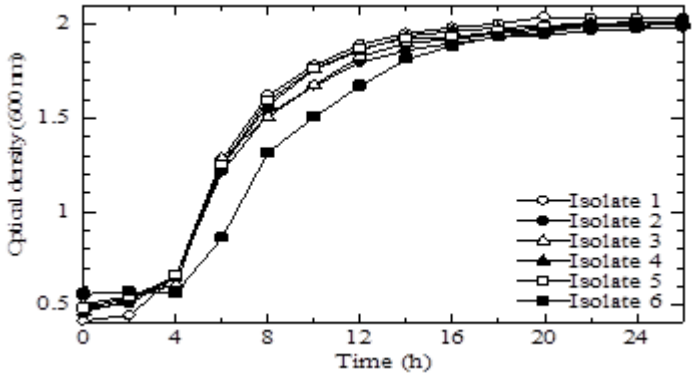

Figure 1: Growth curve of LAB isolates

pH tolerance

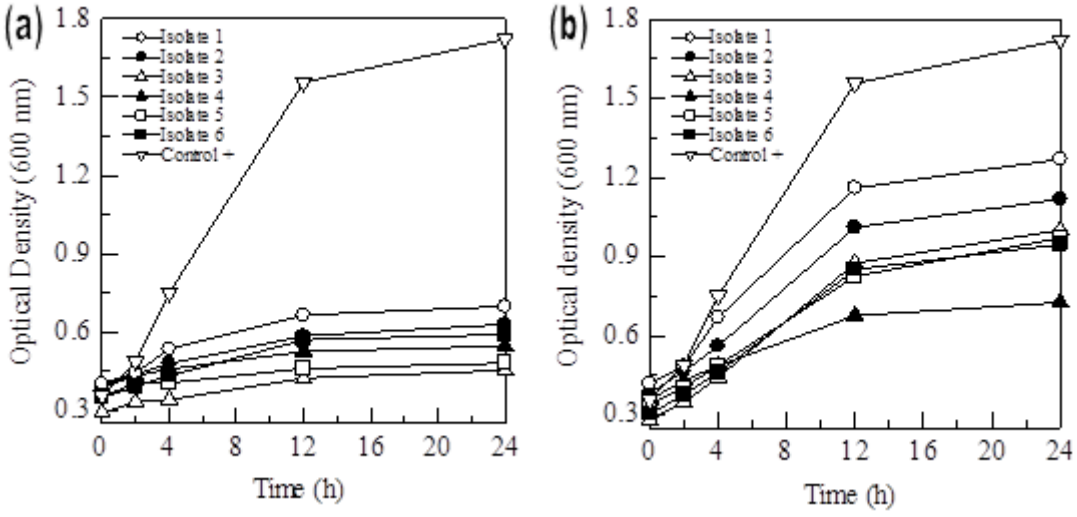

Figure 2: Effect of pH on the growth of LAB: (a) pH 3; (b) pH 4

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In this study, it was observed that LAB has the capability of resistance to acids. At pH 4, the cell density indicated by the absorbance was greater when compared with pH 3. If both are compared to the control, the growth of LAB is strongly influenced by pH (Fig. 2). In order to exert positive health effects, LAB should resist the stressful conditions of the stomach and upper intestine that contain bile [13]. Acidity is believed to be the most detrimental factor affecting the growth and survival of LAB, because their growth falls significantly below pH 4.5 [14]. However, the bacteria are still able to survive and these strains are expected to survive the acidic conditions in fermented food products or the stomach.

Antagonist test using disc diffusion method

The antibacterial activity screening of LAB isolates using disc-diffusion method were showed in Fig. 3. During this investigation, inhibition zone started to appear at 12 hours of incubation time and became more prominent at 24 hours incubation. The inhibition zone however depletes after 36 hours incubation maybe due to death phase of the bacteria thus producing unstable metabolite. The reason of LAB isolates which capable of inhibiting the growth of pathogenic bacteria is that it can produce lactic acid and acetic acid which is acidic to pathogens and thus suppress their growth. This metabolite was exerts by the bacteria during the early phase of their life cycle [15].

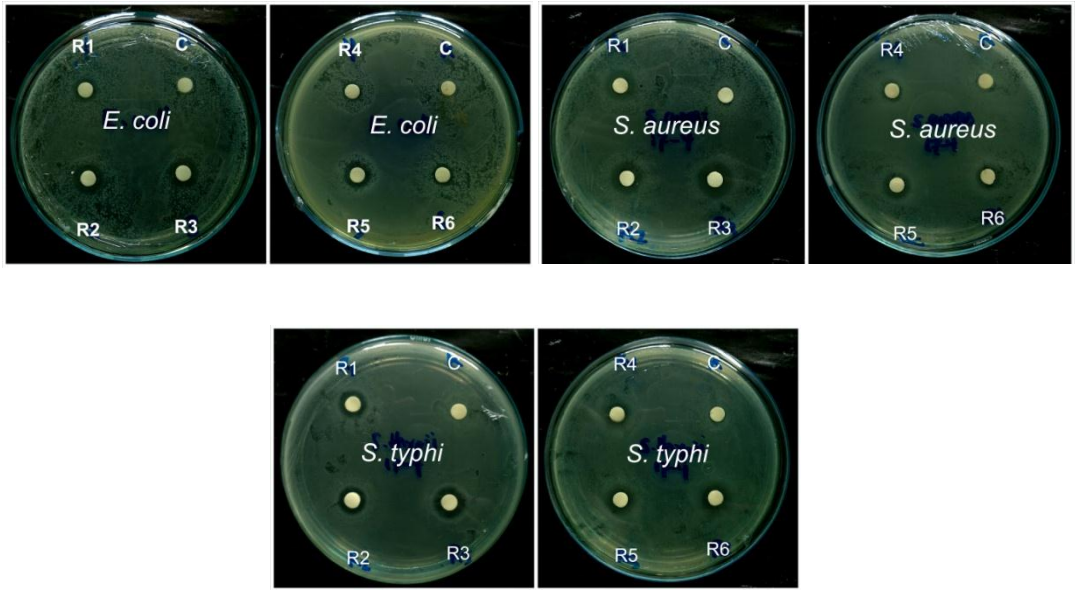

Figure 3: Inhibition zone of LAB isolates against pathogenic bacteria: R1-R6 = isolates, C = negative control

Overall, only three isolates (R2, R3, and R5) showed good antimicrobial activity; ± 12 mm against *E. coli, ±* 13,5 against *S. aureus*, and ± 12 mm against *S. typhi* . Inhibition zone against *S. aureus* (Gram positive bacteria) are greater than on *E. coli* and *S. thyphi* (Gram negative bacteria). Gram-negative bacteria are more resistant to antimicrobials than Gram-positive bacteria due to gram-negative bacteria have several mechanisms of resistance, including the permeability barrier properties of the outer membrane that slows the entry of antimicrobial compounds, as well as the specific mechanisms of resistance that inactivate the compound that prevented penetrate the cytoplasmic membrane or prevent binding to the intracellular side [16].

The antimicrobial activity of lactic acid bacteria may be due to a number of factors. Among these are decreased pH levels, competition for substrates and the production of substances with a bactericidal or bacteriostatic action, including bacteriocins. The drop in pH arising from the production of lactic acid can be enough to inhibit certain strains. This is because the non-dissociated form of lactic acid triggers a lowering of the internal pH of the cell that causes a collapse in the electrochemical proton gradient in sensitive bacteria, hence having a bacteriostatic or bactericidal effect [17].

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16S Ribosomal (rRNA) gene amplification, sequencing and analysis

Isolated DNA was used for amplification of 16S rRNA gene. The process of amplification was using universal primers, 27F and 1525R. The results of the amplification products with a size of ~1,5 kb (Fig. 4) From the results it can be seen that the process of electrophoresis of PCR on R5 isolates did not produce a good PCR product, this could have been caused by DNA, Kit RTG, and primers were not perfectly mixed so annealing process was not running or interrupted. Based on PCR products, only R2 and R3 can be analyzed for nucleotide sequences.

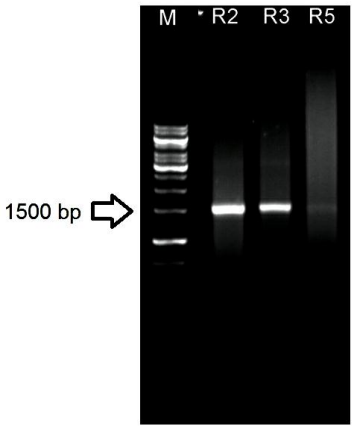

Figure 4: Electrophoresis of PCR products

721 nucleotide sequences obtained for isolates R2 through reverse primer direction. The sequences were analyzed with the data through NCBI BLAST program. Some species of bacteria that has the closest identification value were analyzed by aligning their nucleotide sequences using ClustalW2 online program. The phylogenetic tree was made to determine the closeness of the relationship of species based on their genetic similarities and differences. Based on the phylogenetic tree, isolates R2 has closeness with *Lactobacillus plantarum* FL11-3 (98% identification value and 73% query cover) due to shared ancestor (Fig. 5). While the results of sequence analysis of isolates R3 also shows the closeness with *L. plantarum*, but only has a value of 91% identification.

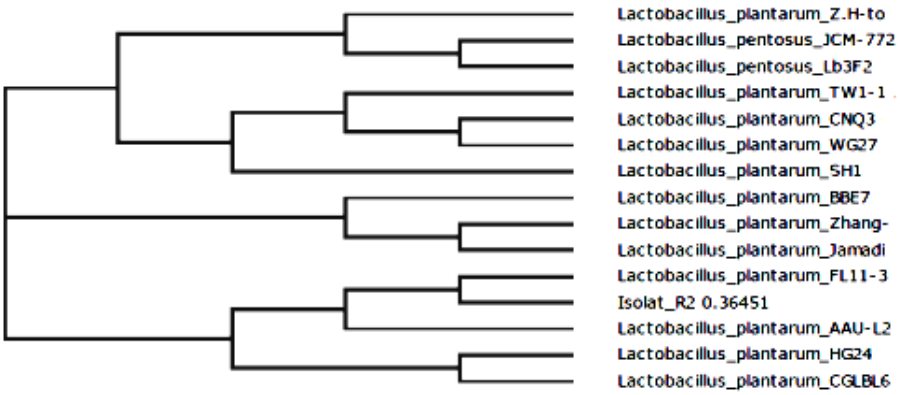

Figure 5: Phylogenetic tree of the isolate R2

CONCLUSION

There are three potential isolates showed good antimicrobial activity against pathogenic bacteria and have resistance to low pH. It showed that the isolates were potentially for probiotic supplement. Sequence analysis showed that LAB isolate was identified as *Lactobacillus plantarum*.

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