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Antibacterial Activity and 16S rRNA Gene Sequencing of Lactic Acid Bacteria from Homemade Fermented Milk in Medan, Indonesia

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A B S T R A C T

Research indicates that fermented products have nutritional and non-nutritional components that can improve health. Milk is commonly used for fermentation products because its rich nutrients support the growth of Lactic Acid Bacteria (LAB). This paper investigates antibacterial activity and 16S rRNA gene sequencing of LAB from homemade fermented milk in Medan City, Indonesia. This paper was an experimental study by In Vitro Models conducted in August-October 2019. This study used three different homemade fermented milk (SF2-4) and positive control of manufactured fermented milk (SF1). All isolated LABs underwent an antibacterial assay by the Disc diffusion method against two pathogens, including *Escherichia coli* (Gram-negative) and *Staphylococcus aureus* (Gram-positive). In addition, there was molecular identification based on 16S rRNA gene sequencing. The zone of inhibition from samples against *Escherichia coli* ranged from 5.600-12.23 mm. The most potent antibacterial activity was found in SF4 (12.23 mm) and the least in SF2 (5.60 mm). Some samples (SF1 and SF2) showed no antibacterial effect against *Staphylococcus aureus* bacteria. The antibacterial activity against *Staphylococcus aureus* bacteria was found only in SF2 and SF3, which were 6.60 mm and 7.14 mm, respectively. Based on the characteristics, enumeration, and antibacterial activity, the authors chose isolated LAB from SF4 for molecular identification based on 16S rRNA gene sequencing. SF4 isolates had a similar 16S rRNA molecule to *Lactobacillus fermentum* strain NBRC 15885 with a homology level of 99.78%. In conclusion, some homemade fermented milk in Medan City, Indonesia, are potential probiotics.

INTRODUCTION

Research indicates that fermented products have nutritional and non-nutritional components that can improve health. Around 90% of fermented products in some countries are produced as homemade products in the form of traditional food and beverages (Tamang *et al.*, 2016). Recently, a study showed the positive role of microbes in the digestive and immune systems of humans (Bansal *et al.*, 2013). Thus, some fermented foods have the potential to make a healthier body.

Food and Agriculture Organization (FAO) and World Health Organization (WHO) have defined probiotics as a group of living microorganisms in adequate numbers that may give some health benefits to the host. A previous study reported that probiotics might prevent and treat some diseases (Khikmah, 2015)**.** The most common type of probiotic for health purposes was Lactic Acid Bacteria (LAB), including *Lactobacillus*, *Leuconostoc*, *Pediococcus*, and *Streptococcus*. LAB secretes a bacteriocin peptide that can inhibit growth and kill some pathogens (Fachrial, Adrian, and Harmileni, 2018).

Milk is commonly used for fermentation products because of its rich nutrients to support LAB growth. However, milk cannot be stored for long and spoil quickly. It can be prevented by pasteurization and

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sterilization. Pasteurization is a method to preserve milk by heating it at a specific temperature below the boiling point of milk. This method keeps the fresh milk's consistency and taste while not eliminating all microbes from the fresh milk (Surono and Hosono, 2011).

Furthermore, the fermentation process promotes the Lactic Acid Bacteria that secrets some antibacterial compounds like organic acids (lactic acid, acetic acid, formic acid), hydrogen peroxide, diacetyl, and bacteriocins. These antibacterial compounds inhibit the growth or even kill bacteria by affecting the metabolism of microbes. *Lactobacillus*, a typical LAB, can hinder the development of some *Enterobacteriaceae* bacteria (*Salmonella* sp, *Escherichia coli*, *Shigella* sp), *Bacillus cereus*, and *Staphylococcus aureus* (Fachrial and Harmileni, 2017; Khristnaviera and Meitiniarti, 2017). This paper investigates antibacterial activity and 16S rRNA gene sequencing of Lactic Acid Bacteria (LAB) from homemade fermented milk in Medan City, Indonesia.

METHOD

This paper was an experimental study by In Vitro Models in Microbiology Laboratory, Universitas Prima Indonesia. In addition, molecular identification was performed at the Indonesia Institute of Science in August-October 2019. This study used three different homemade fermented milk (SF2-4) and positive control of manufactured fermented milk (SF1).

Some materials used in this study included de Man Rogosa and Sharpe Agar (MRSA), de Man Rogosa and Sharpe Broth (MRSB), nutrient agar, 96% alcohol, 70% alcohol, crystal violet solution, safranin solution, 10% hydrogen peroxide solution, aluminium foil, cotton swab, tissue, distilled water, 5M hydrochloric acid solution, Isolated *Escherichia coli* and *Staphylococcus aureus* in slant agar. In addition, this study also used some instruments like a reaction tube, Erlenmeyer flask, analytic scale, petri dish, beaker glass, Durham tube, Bunsen burner, spatula, dropper, inoculation loop, magnetic stirrer, microtube, disc diffusion, autoclave, incubator, callipers, pH meter, antibiotic disc diffusion (Tetracycline, amoxicillin, and ampicillin). The enrichment process was performed by diluting a millilitre sample into 9 ml MRS Broth Media in some reaction tubes. After that, all reaction tubes were homogenized by a vortex and incubated at 37ºC for 18-24 hours in anaerobic conditions (Nasution, Ramadhani, and Fachrial, 2020).

Then, each enriched sample was diluted into different concentrations by serial dilution method until the seventh dilution level. Initially, 0.1 ml of enriched sample was diluted into 0.9 ml of MRSB in a 1.5 ml microtube, and then it was homogenized and labelled as the first dilution level. Furthermore, a hundred microlitres of first-dilution level fermented milk was diluted in the same way as described before until the seventh dilution level (Syukur, Fachrial, and Jamsari, 2014).

One hundred microlitres of diluted samples were cultured into MRSA in some Petri dishes from the fourth to seventh dilution level. Then, all Petri dishes were incubated for 48 hours at 37°C in an incubator. After

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that, the enumeration was performed in each petri dish and expressed as Colony Forming Unit (CFU) per millilitre sample. Furthermore, four quadrant streak methods were used to subculture six random inoculums of LAB colonies into MRS agar in some Petri dishes (Lase *et al.*, 2021).

This study evaluated LAB's morphological and biochemical characteristics from samples. The morphological characteristic included macroscopic and microscopic. Macroscopic characteristics were obtained from observing bacterial colonies on MRS Agar 1% CaCO3 media, including the colonies' shape, color, edges, texture, elevation, and size. Meanwhile, the microscopic characteristic was obtained from the gram staining of the colonies (Nasution, Ramadhani, and Fachrial, 2020; Lase *et al.*, 2021).

Biochemical characteristics analysis of LAB from samples included catalase and fermentation-type test. An inoculum of LAB was applied to an object glass disinfected by alcohol. A drop of 3% hydrogen peroxide solution was dropped into the object glass and observed for the formation of bubbles. Meanwhile, the fermentation-type test was performed by culturing an inoculum of LAB on 10 ml MRSB with a Durham tube placed upside down. Furthermore, the culture was incubated for 24 hours at 37^{c}C. An inoculum of bacteria (*Staphylococcus aureus* and *Escherichia coli*) was suspended in a millilitre of normal saline in a reaction tube and then incubated for 24 hours. Furthermore, it was compared to McFarland Standard 0.5 (Putri, Jannah, and Purwantisari, 2020).

20 ml NA Media was poured into some Petri dishes filled with 1 ml of the bacterial suspension and then homogenized. Some disc diffusion was diffused into the extract, antibiotic, or distilled water and placed at the surface of these media. Each dish was placed in five-disc diffusions, except the antibiotic and distilled water, which only put two disc diffusions. All Petri dishes were incubated at 35-37°C for 18-24 hours. Last, the width of the inhibition zone was measured by a caliper (Mostafa *et al.*, 2018; Mutia, Annisa, and Suhartomi, 2021).

Identification of the 16S rRNA molecule was initially begun by extraction of DNA, followed by amplification and sequencing of the gene. DNA isolation kit (Gene Aid) was used to extract bacterial DNA. The primer used a universal primer (27F: AGAGTTTGATCCTGGCTAG and 1525 R: AGAAAGGAGGTGATCCAGCC). Meanwhile, the amplification used a PCR solution containing primer DNA, PCR-Grade water, and KAPA Taq extra hot start the ready mix with dye. Initially, the PCR process began in a pre-denaturation process at 95°C for five minutes, followed by denaturation at 94°C for a minute, annealing at 56°C for a minute, and extension at 72°C for 1.5 minutes, and the final extension for 72°C for five seconds. Finally, the amplified gene was separated into a 1.5% agarose gel with EtBr staining, and the gel was observed under the GelDoc Machine (BioRad). This amplified gene was then sequenced by a one-way primer reverse performed by the Indonesian Institute of Sciences. The Basic Local Alignment Search Tool (BLAST) program analyzed and compared the obtained sequence to the NCBI database (Fachrial and Harmileni, 2018; Siburian *et al.*, 2021).

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RESULT

Based on Table 1, the number of colonies in SF1, 2, 3, and 4 sequentially was 1, 117, 139, and 3. The number of colonies was used to determine the enumeration of LAB in all fermented milk samples. The enumeration of LAB in SF1, 2, 3, and 4 sequentially were 1.0 x 10^6 CFU/ml, 117 x 10^5 CFU/ml, 139 x 10^5 CFU/ml, and 3 x 10^7 CFU/ml.

Table 1. Enumeration of LAB from Homemade and Manufactured Fermented Milk

Sample	Dilution	Number of Colonies
SF 1	10^{-6}	1
SF 2	10^{-5}	117
SF 3	10^{-5}	139
SF 4	10^{-7}	3

The isolated LAB from samples underwent the evaluation of morphological and biochemical characteristics. The morphological characteristics included the evaluation of macroscopic and microscopic. Figure 1 shows the macroscopic characteristics. The macroscopic appearance of the lactic acid bacteria colonies from all samples was a creamy white LAB colony.

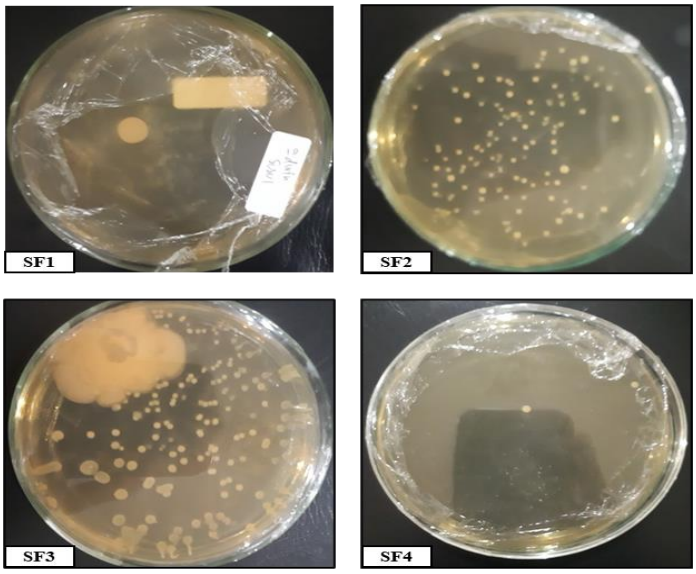

Figure 1. Macroscopic Morphology of Lactic Acid Bacteria Colonies from all Samples on MRSA Media

Figure 2 shows the microscopic view with gram staining. LAB in SF1 was gram-positive cocci bacteria, while in SF2-SF4 was gram-positive bacilli. Furthermore, this study also identified the biochemical characteristic of LAB in these bacteria. Bacteria in SF2-SF4 showed no catalase activity but a heterofermentative activity. Meanwhile, the positive control (SF1) showed a catalase and heterofermentative activity.

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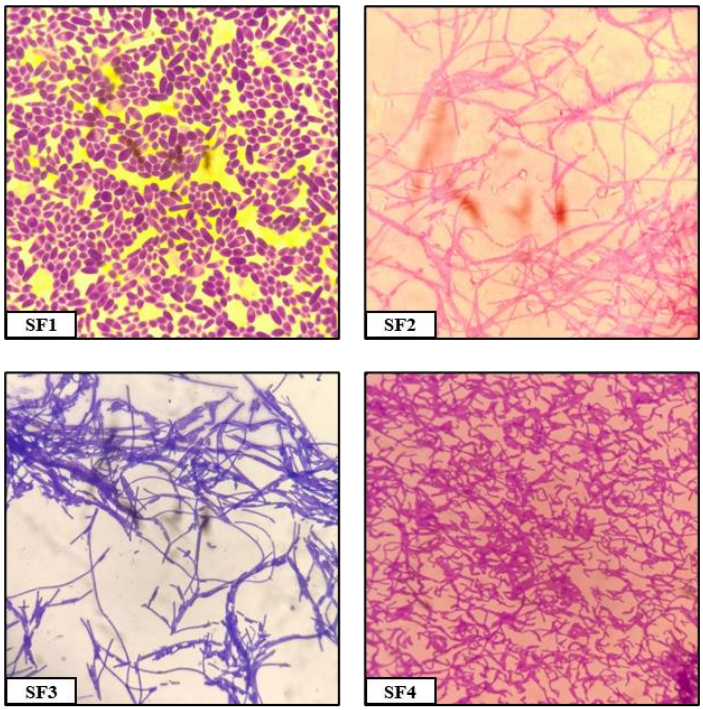

Figure 2. Microscopic View of LAB in all samples. Staining: Gram Staining. Magnification: 1000x

All isolated LABs underwent an antibacterial assay against two different pathogens, including *Escherichia coli* (Gram-negative) and *Staphylococcus aureus* (Gram-positive), by Disc diffusion method, and the result of the antibacterial activity of LABs against these pathogens was described in Table 2. Table 2 shows that the zone of inhibition from samples against *Escherichia coli* ranged from 5.600-12.23 mm (including disc diameter, 5.2 mm). The most potent antibacterial activity was found in SF4 (12.23 mm) and the least in SF2 (5.60 mm). On the other hand, some samples (SF1 and SF2) showed no antibacterial effect against *Staphylococcus aureus* bacteria. The antibacterial activity against *Staphylococcus aureus* bacteria was found only in SF2 and SF3, which were 6.60 mm and 7.14 mm, respectively.

Table 2. Antibacterial Activity of LAB from Samples against Two Different Pathogens

Sample	The zone of inhibition (mm)	
	Escherichia coli	*Staphylococcus aureus*
SF 1	6.98 mm	-
SF 2	5.60 mm	6.60 mm
SF 3	7.20 mm	7.14 mm
SF 4	12.23 mm	-

Based on the characteristics, enumeration, and antibacterial activity, the authors chose isolated LAB from SF4 for molecular identification based on 16S rRNA gene sequencing. Figure 3 shows DNA sequences.

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Consensus_ SF4

TTGATTGATGGTGCTTGCACCTGATTGATTTTGGTCGCCAACGAGTGGCGGACGGGTGAG
TAACACGTAGGTAACCTGCCCAGAAGCGGGGGACAACATTTGGAAACAGATGCTAATACC
GCATAACAACGTTGTTCGCATGAACAACGCTTAAAAGATGGCTTCTCGCTATCACTTCTG
GATGGACCTGCGGTGCATTAGCTTGTTGGTGGGGTAATGGCCTACCAAGGCGATGATGCA
TAGCCGAGTTGAGAGACTGATCGGCCACAATGGGACTGAGACACGGCCCATACTCCTACG
GGAGGCAGCAGTAGGGAATCTTCCACAATGGGCGCAAGCCTGATGGAGCAACACCGCGTG
AGTGAAGAAGGGTTTCGGCTCGTAAAGCTCTGTTGTTAAAGAAGAACACGTATGAGAGTA
ACTGTTCATACGTTGACGGTATTTAACCAGAAAGTCACGGCTAACTACGTGCCAGCAGCC
GCGGTAATACGTAGGTGGCAAGCGTTATCCGGATTTATTGGGCGTAAAGAGAGTGCAGGC
GGTTTTCTAAGTCTGATGTGAAAGCCTTCGGCTTAACCGGAGAAGTGCATCGGAAACTGG
ATAACTTGAGTGCAGAAGAGGGTAGTGGAACTCCATGTGTAGCGGTGGAATGCGTAGATA
TATGGAAGAACACCAGTGGCAAGGCGGCTACCTGGTCTGCAACTGACGCTGAGACTCGAA
AGCATGGGTAGCGAACAGGATTAGATACCCTGGTAGTCCATGCCGTAAACGATGAGTGCT
AGGTGGTTGGAAGGGTTTCCGCCCTTCAGTGCCGGAGCTAACGCATTAAGCACTCCGCCT
GGGGAGTACGACCGCAAGGTTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTG
GAGCATGTGGTTTAATTCGAAGCTACGCGAAGAACCTTACCAGGTCTTGACATCTTGCGC
CAACCCTAGAGATAGGGCGTTTCCTTCGGGAACGCAATGACAGGTGGTGCATGGTCGTCG
TCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTTACTAG
TTGCCAGCATTAAGTTGGGCACTCTAGTGAGACTGCCGGTGACAAACCGGAGGAAGGTGG
GGACGACGTCAGATCATCATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGGACG
GTACAACGAGTCGCGAACTCGCGAGGGCAAGCAAATCTCTTAAAACCGTTCTCAGTTCGG
ACTGCAGGCTGCAACTCGCCTGCACGAAGTCGGAATCGCTAGTAATCGCGGATCAGCATG
CCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGAGAGTTTGTA
ACACCCAAAGTCG

Figure 3. DNA Sequence of SF4 LAB Isolate

Figure 3 shows the 16S rRNA gene sequencing from isolated LAB in SF4. This data was trimmed and assembled for analysis in the BLAST registered with National Center for Biotechnology Information (NCBI). The analysis result was a phylogeny tree shown in Figure 4.

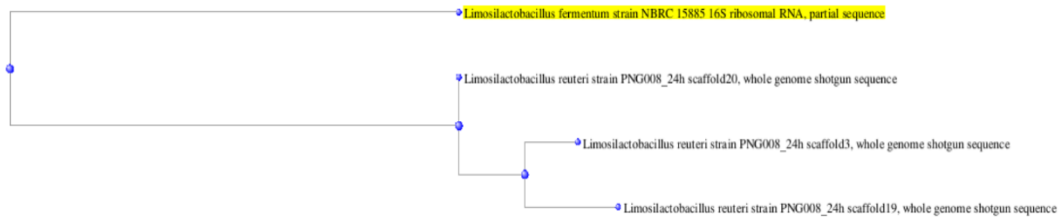

Figure 4. Phylogenetic tree of SF4 isolates

Based on Figure 4, SF4 isolates had a similar 16S rRNA molecule to *Lactobacillus fermentum* strain NBRC 15885 with a homology level of 99.78%.

DISCUSSION

This paper found that the enumeration of LAB in SF1, 2, 3, and 4 sequentially were 1.0 x 10^6 CFU/ml, 117 x 10^5 CFU/ml, 139 x 10^5 CFU/ml, and 3 x 10^7 CFU/ml (Table 1). It showed a similar result to Khalil and Anwar (2016), who reported that the enumeration of LAB under aerobic conditions ranged from 1.1 x 10^5 to 7.2 x 10^8 CFU/ml, and anaerobic went from 6.1 x 10^5 to 5.3 x 10^7 CFU/ml (Khalil and Anwar, 2016). In addition, the macroscopic appearance of the lactic acid bacteria colonies from all samples was a creamy white LAB colony (Figure 1). It showed a similar result to Khalil and Anwar (2016) and Siburian *et al.* (2021), which reported that the LAB colony in MRS Agar 1% CaCO3 media was an appearance as a white

colony with a smooth surface (Siburian *et al.*, 2021). Moreover, the microscopic view with gram staining showed that LAB in SF1 was gram-positive cocci bacteria, while in SF2-SF4 was gram-positive bacilli. It was similar to Khalil and Anwar (2016), who reported that LAB was characterized as either bacilli or cocci gram-positive (Khalil and Anwar, 2016).

Antibacterial activity classifications are based on the wide inhibition zone. Morales *et al*. (2003) grouped the antimicrobial activity based on the wide inhibition zone into four categories: weak activity (5 mm), moderate (5–10 mm), strong (>10–20 mm), and very strong (>20–30 mm). Thus, most samples in this study showed a moderate antibacterial effect against these pathogens. However, SF4 showed a strong antibacterial effect against *Escherichia coli* but no antibacterial effect against *Staphylococcus aureus*. It showed a similar result to Zaraswati *et al*. (2017), who reported that some LAB strains from fermented milk had some antimicrobial activities against *Escherichia coli* (19.83 mm) and *Candida albicans* (19.33 mm).

Some studies have investigated the mechanism of action of antibacterial effects from fermented milk. Fermented milk contains *Lactobacillus bulgaricus, Lactobacillus acidophilus, and Lactobacillus casei* (Morales *et al.*, 2003; Dwyana, 2017). Prior studies reported that LAB had some compounds responsible for antibacterial effects, including bacteriocin and lactic acid. Bacteriocin is a high molecular weight (> 30 kDa) of heat-labile proteins; this protein is a group of endopeptidases that lyse peptidoglycan in bacterial cell walls. Zhu and Zhang (2020) reported that Lacidophilin, a bacteriocin, could disturb the integrity of the bacterial cell membrane through lipid peroxidation and cell oxidative damage. It leads to the leakage of electrolytes, nucleic acid, and proteins. It also restrained phosphorus metabolism, inhibited the growth of the bacteria, and caused changes in some bacterial proteins. In addition, Ijaz (2021) also reported that LAB might also secrete lactic acid that reduces the medium pH and inhibiting the growth of various enteropathogens and foodborne pathogens (Zhu and Zhang, 2020; Ijaz, Iqbal and Saeed, 2021).

Furthermore, this paper revealed that SF4 isolates had a similar 16S rRNA molecule to *Lactobacillus fermentum* strain. *Lactobacillus fermentum* predominantly ferments some fermented dairy products and human microbiota. Ijaz et al. (2021) reported that the dairy samples from some rural areas in Southern Punjab, including cow milk and fresh homemade yogurt, had some isolates contained *Lactobacillus fermentum* by 16S rRNA sequencing, which may be a potential source of probiotics. On the other hand, Allaith et al. also reported that other fermented beverages like malt milk (Boza) and apple juice (Sider) might have 16S rRNA molecule that was identic *Limosilactobacillus fermentum* (also known as *Lactobacillus fermentum*) and *Leuconostoc mesenteroides*, with a similarity level of 99.8%–100.0% (Ijaz, Iqbal and Saeed, 2021; Allaith *et al.*, 2022).

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CONCLUSION

In conclusion, some homemade fermented milk in Medan City, Indonesia, are potential probiotics. They had Lactic Acid Bacteria with moderate antibacterial activity against gram-positive and gram-negative bacteria. One was identified as *Lactobacillus fermentum* strain NBRC 15885 with a homology level of 99.78% based on 16S rRNA gene sequencing.

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