Antimicrobial Screening of Lactic Acid Bacteria Isolated from Fermented Milk Buffalo (Dadih)

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Abstract- *Dadih is a traditional Indonesian buffalo milk product that is fermented naturally and that is believed to have many benefits to human health, this can be attributed to the nature of the lactic acid bacteria involved in the fermentation process. In the fermentation process, lactic acid bacteria alter complex organic molecules compounds such as proteins, carbohydrates and fats into simpler molecules, easily soluble and high digestibility. In this study, antimicrobial activity was performed by diffusion method in order to fight the pathogenic bacteria Salmonella enteritidis strain ATCC BAA-711, Salmonella enteritidis strain local Malaysia code number 4301/15, Escherichia coli strain ATCC 35401and Staphylococcus aureus strain ATCC 2592. Antimicrobial activity of lactic acid bacteria originated from Kerinci dadih, Lactobacillus plantarum showed strong antimicrobial activity against Salmonella enteritidis strain local Malaysia code number 4301/15 (±16,0 mm), Salmonella enteritidis strain ATCC BAA-711 (± 12,0 mm), Escherichia coli strain ATCC 35401 (± 11,0 mm) and Staphylococcus aureus strain ATCC 25923 (±12,0 mm). While Lactobacillus delbruekki showed lower antimicrobial activity against Salmonella enteritidis strain local Malaysia code number 4301/15 (± 14,0 mm), Salmonella enteritidis strain ATCC BAA-711 (±10,0 mm), Escherichia coli strain ATCC 35401 (± 10,0 mm) and Staphylococcus aureus strain ATCC 25923 (± 11,5 mm).*

Keywords: Antimicrobial activity; Lactobacillus plantarum; Lactobacillus delbrueckii.

I. Introduction

Dadih is a buffalo milk product that is naturally fermented and that is a very famous traditional Indonesian special food and quite popular. This food is only found in the Sumatra islands, particularly in province West Sumatra, Riau and Jambi. In rural communities, dadih is often consumed directly or with rice after being given a shallot slice and red chillies or mixed in cold drinks with ketan emping, coconut milk and brown sugar. According Sugitha (1995), dadih is consumed as side dish, interlude food, complementary ceremonies and traditional medicine. In Jambi province, the dadih can be found in the Kerinci district, which includes the regions of Gunung Kerinci, KayuAro and Air Hangat. The dadih produced in Kerinci district are similar with dadih which area produced in West Sumatra province and other regions.

In Kerinci district the process of making dadih from buffalo milk has long been known by the community and done through the hereditary without repairing its processing techniques. The process of dadih making is traditionally done by not giving heat to buffalo milk and not using starter culture. Raw and fresh buffalo milk is stored in a plastic bucket, filtered with cloth, then placed in the bamboo tube as much as + 250 ml and covered with banana leaves that have been withered over on fire and then it is naturally fermented at room temperature for 24-48 hours until formed a clump that resembles milky-white colored pasta which has solid and smooth texture, sour taste and a distinctive aroma (Surono and Hosono, 1995; Surono and Hosono, 2000). Nutrition content of buffalo dadih varies, depending on the area of production and type of buffalo milked. Dadih Kerinci has water content around 69.08 - 73.12%, protein 5.65 - 5.78%, fat 7.94 - 8.27% and acid content 0.96-1.03% (Afriani, 2008). Dadih also contains 16 amino acids (13 essential amino acids and 3 non-essential amino acids) so it can be nutritious foods that are easily absorbed by the body and vitamin A 1.70-7.22 IU/g (Yudoamijoyo et al. 1983). The type of bamboo used for making dadih, among others bambu gombong (Gigantochloaverticillata), bambu ampel (Bambusa vulgaris), bambu talang (Schizostachyum brachycladum) and bambu betung (Dendrocalamus asper). Bamboo used as an old picked container that has relatively low water content, so it can produce good dadih quality. In making the bamboo cut with a high size between 20-30 cm and diameter of 5-8 cm and the top of the perforated hole is slightly roughly as big as a finger. Further cleaned and reversed to remove the dirt contained in the tube (Zulbardi, 2003). The type of bamboo used is hygroscopic, has a bitter

taste that can prevent the product from ants and have several types of microbes that naturally can ferment the milk into dadih (Usmiati et al. 2011).

Natural fermentation process in the dadih making involves different types of microbes found on the surface of the inner bamboo tubes, cover the leaf surface and from buffalo milk used (Usmiati and Risfaheri, 2013). The fermentation process involves a number of Gram-positive bacteria among others Lactobacillus plantarum, Lactobacillus brevis, Streptococcus agalactiae, Bacillus cereus and Streptococcus uberis and Gram-negative bacteria among others Escherichia coli and Klebsiella sp (Pato, 2003). Lactic acid bacteria, especially the genus Lactobacillus and Bifidobacteriaceae, are the most of microbes widely used in the food and pharmaceutical industry (Piano et al. 2006). Lactic acid bacteria found in dadih is believed to have many benefits to human health, this can be attributed to the nature of the lactic acid bacteria (LAB) involved in the fermentation process. In the fermentation process, lactic acid bacteria alter complex organic molecules compounds such as proteins, carbohydrates and fats into simpler molecules, easily soluble and high digestibility. The simple molecular compounds produced by lactic acid bacteria consist of various types of low molecular weight components, including organic acids, alcohols, carbon dioxide, diacetylides, hydrogen peroxides, acetaldehyde and other metabolites. Many metabolites have a broad spectrum of activity against other species and the production is largely influenced by the food matrix itself. An important attribute of lactic acid bacteria is its ability to produce antimicrobial components, especially potential bacteriosines into biopreservatives to replace chemical preservatives on food ingredients to extend the life of the product. The bacteriocins ability to perform activity it's biopreservative is achieved by the effects of growth inhibition on dangerous pathogenic microorganisms (Savadogo et al. 2006). This study aims to screen the antimicrobial of lactic acid bacteria isolated from fermented milk buffalo (dadih).

II. METHOD AND MATERIAL

Samples

Fermented buffalo milk (dadih) was obtained from conventional buffalo farms in Kerinci District, Jambi Province- Sumatera Indonesia as a local source of lactic acid bacterial strain. Testing was carried out in the form of isolation and identification of lactic acid bacteria derived from dadih. Isolation was done using de-Man's Rogosa Sharpe media (MRS, OxoidUK) and identification of lactic acid bacteria includes morphological characteristics and molecular identification.

Isolation of lactic acid bacteria

Isolation of lactic acid bacteria derived from the dadih Kerinci done in according to the method Hayakawa 1992; Adnan and Tan, 2007 and Khedid et al., 2009. The isolation of lactic acid bacteria was carried out by suspending 10 grams of dadih (48 hours fermentation) to 90 mL 0.85% NaCl (dilution 10-1) and serial dilution until 10-8, then pipette 0.1 mL from dilution 10-2 to 10-8is inoculated into the de-Man's Rogosa Sharpe Agar (MRS A) plate containing purple bromo cresol (BCP) 0, 01% and anaerobally incubated for 48 hours at 37°C for bacterial growth.

Colonies observed with flat sightings and yellow or gray to brown around the colony. Colonies of different colors and sizes are recycled back into the same media as quadrant scratches and incubated is performed under the same conditions as above. Scratching is done until a single and uniform colony is obtained and colonies that already pure are chosen and wound up on the MRS agar for further identification. For stock culture, pure colony was grown for 2 days in the semi solid MRS agar (0.7% agar) containing 0.2% CaCO3 in the form of agar and stored at 4 oC or pure colonies grown in MRS broth containing 20% glycerol stored at -80 ^{o}C.

Identification and characterization of isolates of LAB

Identification of lactic acid bacteria isolates include phenotype and genotype characteristics. Phenotype identification based on morphology characteristics and genotype identification based on molecular analysis using 16S rRNA.

Morphological characteristic for LAB identification

Identification and characteristic of colonies include size, pigmentation, shape, edge and elevation, while cell morphological characteristics include gram staining and spore staining. Colonies observed with flat sightings and yellow or gray to brown around the colony. Colony observations include (1) size consists of small, medium and large, (2) pigmentation, (3) the edges form consisting of circular, irregular and rhizoid, and (4) the elevation consists of flat, raised, convex and umbonate (Cappuccino and Sherman, 2001; Sunatmo, 2007).

Gram Staining

Gram staining begins with dropping the main dyes (crystal violet), evenly above the culture on the glass object and stayed for 1 minute. Then the object glass is tilted to remove the advantage of crystal violet, then rinsed with aquadest flows carefully. Furthermore, it was dropped with lugol for 2 minutes and tilted the object glass as above, then rinsed with aquadest flows carefully, the remaining color is cleaned with ethanol 95%, drops 10-20 seconds until the color of the crystal is no longer flowing from the glass of the object. Then washed back with the flowing aquadest, then drained and then drip with safranin solution for 10 - 20 seconds. The object glass is tilted and rinsed back with the flowing aquadest, then drained and remaining waste water is absorbed by absorbing paper. Preparations are ready to be observed with a microscope.

Observation with a microscope is performed using an objective lens of immersion oil (1000x), starting with the lowest magnification and gradually being replaced. Observation was done on size, form, method of grouping (single, couple, chain, clustered, etc). The

positive gram reaction is characterized by purple cell color or blue cell colors and negative pink muda (Hadioetomo, 1993; Cappuccino and Sherman, 2001; Sunatmo, 2007).

Spore staining

Similar to gram staining, as much as one loop sterile aquadest was placed on a clean glass object. A small amount of isolate was then transferred to the glass by using a sterile ose needle to mix and spread the isolate evenly on the glass to air dry. The sample was saturated with malachite green stain solution and heated for 2-3 minutes making sure to prevent any despoil or boil. Counter staining with safranin solution for 30 seconds begins after the sample has been rinsed with aquadest flows carefully and drained. At last, the object glass was tilted, rinsed with flowing aquadest, and drained. The remaining waste water was absorbed by absorbing paper. Preparations were ready to be observed with a microscope. Observation with a microscope was performed using an objective lens of immersion oil (1000x), starting with the lowest magnification and gradually being replaced (Sunatmo, 2007).

Identification of Lactobacillus isolates using 16S rRNA, sequencing and analysis

The 16S rRNA molecular DNA sequencing technique was performed on the isolates (Sambrook and Russel, 2006), including genomic DNA extraction, DNA electrophoresis, gen amplification and analysis of base sequence 16s rRNA genes.

Genomic DNA extraction

DNA genomic isolates of selected lactic acid bacteria were extracted using Ref. 740952.50 NucleoSpin® TissueLot.number 1397/009 (Macherey-Nagel, German) according to manufacturer's instructions. Bacterial isolates are cultured in 10 ml MRS broth, shaking in the incubator shaker at 150 rpm, 37oC overnight. A total of 1.5 mL of bacterial culture was centrifuged at 8,000 x g, 4 oC for 5 minutes, then the supernatant is removed, and the liquid is removed as much as possible. For cell lysis, the bacterial pellet is suspended in 180 µl buffer T1 by pressing the pipette up and down until no clumps remain. Then 25 µl of proteinase K was added, fast vortex and the sample was shaken in the incubator shaker at 56 oC for 3 hours until the lysis was formed. Sample vortex and then added 200 µl of buffer B5. Sample vortex returns and incubated at 70 oC for 10 minutes, centrifuge for 10 minutes at 11.000 x g speed, and the supernatant is transferred to a sterile new microtube. For DNA binding, into the sample was added 210 µl ethanol (96-100%) and vortex to homogeneous. Then carefully sample is placed in NucleoSpin @ Tissue Column into sterile collection tubes and centrifuge for 1 minute at 11, 000 xg. Incoming stream is discarded, and the column is placed back into sterile collection tube (2 mL). To wash the cell, buffer BW heated up to 50 ºC, then 500µl is added to the column and centrifuge for 1 minute at 11,000 x g. Incoming stream is discarded, and the column is placed back into sterile collection tube and followed by a laundering step with a buffer B5. 600µl buffer B5 added and centrifuged for 1 minute at 11,000 x g. Incoming stream is discarded, and the column is placed back into sterile collection tube. Then dry out silica, columns were centrifuge for 1 minute at 11,000 x g and collection tube removed. For DNA elution, the nucleoSpin @ Tiissue Column is placed on sterile microcentrifuge tube 1.5 mL. Then 100 µl buffer BE is added, incubate at room temperature for 1 minute and centrifuge for 1 minute at 11,000 x g. Then, all purified DNA samples are stored at -20 ºC or 70 ºC for subsequent use of the process.

Electrophoresis

Genome DNA was confirmed using Major Science Mini Horizontal Gel Electrophoresis (USA). 1 µl loading dyeand 5 µl of DNA samples were mixed homogeneously on parafilm, then use the micropipette placed into well gel 0.7 % agarose for electrophoresis, while the marker was used as much as 3 µl mixed with 1 µl loading dye homogeneously and placed in different wells. Electrophoresis was performed for 60 minutes with voltage 85 volt in buffer solution TBE 1X, then the gel is stained with red gel staining for 5 to 10 minutes, then rinsed with aquades for 10 minutes. Then bands are seen using UV trans-illuminators and gel is stored at 4 ºC.

Gen amplification

The selected 16S rRNA universal primers: 27Forwad (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492Reverse (5'-GGTTACCTTGTTACGACTT -3') used to amplify the 16S rRNA region (Marchesi et al., 1998; Zhang, et al., 2007) and amplification process using PCR AB Applied Biosystem verity 96 Thermal Cycler machine (Fisher Scientific, USA). The PCR reaction was made in a total volume of 25 µl with a final concentration of 1X, consists of 12.5 of Econotaq Plus Green 2x Master Mix, 0.25 µl of Forward primer (100 µM), 0.25µl of Reverse primer (100 µM), 1.0 µl of DNA Template (10 ng/ µL) and 11.0 µl of Water nuclease free. All constituents of the mixture were placed in the thin wall of PCR tube and vortex briefly with microcentrifuge, then run using PCR machine.

PCR conditions are performed as follows pre heat thermocycler up to 94 oC and then with initial denaturation of DNA at an initial temperature of 94 oC for 2 minutes. Then proceed with 30 cycles of denaturation at 94 oC for 30 seconds, annealing at 50oC for 30 seconds, extension at 72oC for 30 seconds and final extension at 72ºC for 10 minutes before cooling down to 4ºC (EconoTaq ® PLUS GREEN 2X Master Mix – Lucigen). All the positive colonies which amplified via colony PCR were analysed through 0.7% agarose gel electrophoresis. Furthermore, the gel is stained with red gel staining and bands are seen using UV trans-illuminators. Purification of PCR products and sequencing is done by a sequencing service company. The complete 16S rRNA DNA sequence was built using contig assembly by VectorNTi (Invitrogen, USA) and compared with other DNA sequences in the Genbank database using BLASTN program

(http://www.ncbi.nlm.nih.gov/BLAST/). Homology sequences over 97% are considered to have the same species. To determine the phylogenetic relationship, the 16S rRNA sequence is aligned using Clustal W (Thompson et al. 1994).

Antimicrobial activity of lactic acid bacteria against pathogenic bacteria

The Kirby-Bauer disc diffusion assay is carried out based on recommendations given the Clinical Laboratory Standards Institute, CLSI (Jorgensen and Turnidge 2015). Pathogenic bacteria used in this experiment is a bacterium causing gastrointestinal disorders system among others Salmonella enteritidis strain ATCC BAA-711, Salmonella enteritidiS local Malaysia (code number : 4301/15), Escherichia coli strain ATCC 35401 and Staphylococcus aureus strainATCC 25923 merupakan koleksi dari University Malaya Bacteriology and Toxicology Laboratory, Institute of Biological Sciences, Faculty of Science, University of Malaya, Malaysia. 6 selected lactic acid bacteria were grown in de-Man'sRogosa Sharpe broth (MRSB) and anaerobically incubated for 24 hours at 37 ºC. Pathogenic bacteria (Salmonella enteritidis strain ATCC BAA-711, Salmonella enteritidiS local Malaysia (code number : 4301/15), Escherichia coli strain ATCC 35401 and Staphylococcus aureus strainATCC 25923) grown in Mueller Hinton broth at 37 ºC for 24 hours. Pathogenic bacteria were standardized with an initial population of at least 108 cfu/mL (standard McFarland no.2) in the Nutrient Broth medium, then diluted in physiological NaCI until the population reaches 105 cfu / mL before use in the confrontation test.

In the petridish the size of 100x200 mm pour Mueller Hinton agar sterile to 4 mm thick and leave to freeze. Then pathogenic bacteria are applied to the surface Muller Hinton agar so to use the sterile cotton swab. 50 µl of each lactic acid bacterial culture impregnated into 6 mm in diameter sterile, commercial blank disc (GE healthcare life sciences whatman grade AA and left to dry. Antibiotic amoxicillin as a positive control and sterile broth MRS as a negative control is also applied to discs of much 50 µl. All discs were ensured to be fully dried before application on bacterial lawn. All discs are applied the agar using sterile forceps and dipressed gently to ensure uniform contact. The discs were placed 8 per plate (positive and negative control discs included) equidistantly to avoid the overlapping of zones of inhibition. The plates were inverted and incubated at 37 ºC for 18 to 24 hours. Any compound that diffused from the discs that can inhibit bacterial growth will make it presence with the development of the clearing zone (inhibition zone). If present, their diameters were measured to the nearest whole millimeter with a caliper against dark. Non-reflective background. The assays were carried out in triplicates and the mean diameter of zone was calculated. The same is done for testing further antimicrobial activity.

III. RESULTS

Isolation of lactic acid bacteria

There are twenty-nine colonies obtained from dadih kerinci have different characteristics consisting of size, pigmentation, shape, edge and elevation are presented in Table 1 and Figure 1.

Table 1. Characteristic morphology of colony of lactic acid bacteria origin of dadih

No.	Isolate Code	Size	Pigmentation	Shape	Edge	Elevation
1.	B1.1,B1.2,B2.1.A,B2.1.B,B2.1.C,B4.1.1,B4.2.1,B4.3.1,B4.3.2,B7.B,B8.1.1,B8.2.1,B8.3.1,B8.3.3A,B9. A, B9. B, B11.B	small	milky white or cream	circular	entire	convex
2.	B6.1.1. B,B7. A,B8.1.2, B8.3.2,B10.1,B11.A	medium	milky white or cream	circular	entire	convex
3.	B2.2,B3. A,B4.3.3,B5.2,B6.1.1. A,B6.2	big	milky white or cream	circular	entire	convex

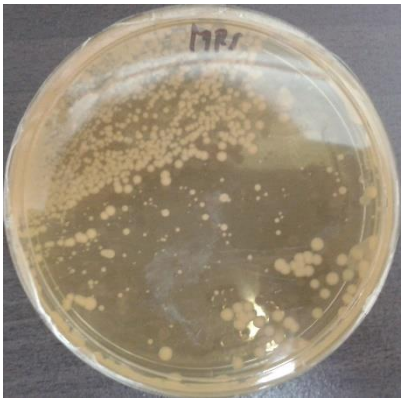


Figure 1. Colonies of LAB origin from dadih

Morphological characteristic for LAB identification

Bacterial staining is performed to distinguish morphological characteristics and cell structure of lactic acid bacteria. The morphological characteristic of isolate cells of lactic acid bacteria are presented in Table 2 and Figure 2.

Table 2. Cell morphological characteristic isolates of lactic acid bacteria origin dadih

No	Isolate Code	Staining		Cell morphology	Genus
		Gram	Spore		
1.	B1.1, B2.1.C, B4.3.2, B8.1.1, B8.3.1, B9. A, B9. B	+	-	Bacilli, short, smalll	Lactobacillus
2.	B1.2, B2.1.A, B2.1.B, B2.2, B3.A, B4.1.1, B4.2.1, B4.3.1, B4.3.3, B5.2, B6.1.1.A, B6.1.1.B, B6.2, B7.A, B7.B, B8.1.2, B8.2.1, B8.3.2, B8.3.3A, B10.1, B11.A, B11.B	+	-	Bacilli, long, large	Lactobacillus

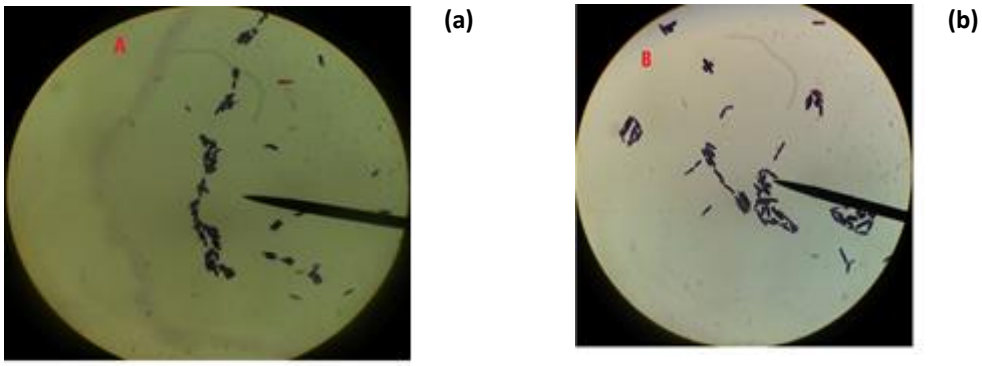


Figure 2. Cell morphology of LAB of dadih (1000 X)

Identification of Lactobacillus isolates using 16S rRNA, sequencing and analysis.

Based on 16S rRNA gene sequence analysis result of lactic acid bacteria isolates origin dadih using BLAST program two species of Lactobacillus were obtained among them Lactobacillus plantarum and Lactobacillus delbrueckii (Table 3).

Table 3. The analysis result BLAST of the isolate of lactic acid bacteria origin of dadih

No.	Isolate code	Description	GenBank Accession Nomor	Query coverage	Max Ident
1.	B.1.1	*Enterococcus faecalis*	KJ725203.1	100%	100%
2.	B.1.2	*Klebsiella pnuemoniae*	CP006656.1	100%	99%
3.	B.2.1A	*Lactobacillus plantarum*	KJ725205.1	100%	100%
4.	B.2.1B	*Lactobacillus plantarum*	KR816164.1	100%	100%
5.	B.2.1C	*Enterococcus faecalis*	CP004081.1	100%	100%
6.	B.2.2)	*Enterococcus faecalis*	CP004081.1	100%	99%
7.	B.3A	*Enterococcus faecalis*	KT260534.1	100%	100%
8.	B.4.1.1	*Enterococcus faecalis*	KT260534.1	100%	99%
9.	B.4.2.1	*Lactoacillus plantarum*	KR816164.1	100%	99%
10.	B.4.3.1	*Lactoacillus plantarum*	KR816164.1	100%	100%
11.	B.4.3.2	*Klebsiella pnuemoniae*	CP013322.1	100%	100%
12.	B.4.3.3	*Enterococcus faecalis*	CP004081.1	100%	99%
13.	B.5.2	*Klebsiella pnuemoniae*	CP006656.1	100%	99%
14.	B.6.1.1A	*Enterococcus faecalis*	KJ725203.1	100%	100%
15.	B.6.1.1B	*Lactobacillus delbrueckii*	KJ725217.1	100%	100%
16.	B.6.2	*Lactoacillus plantarum*	KR816164.1	100%	100%
17.	B.7A	*Enterococcus faecalis*	CP004081.1	100%	100%

18.	B.7B	*Enterococcus faecalis*	KT260534.1	100%	100%
19.	B.8.1.1	*Enterococcus faecalis*	KT260534.1	100%	99%
20.	B.8.1.2	*Klebsiella pnuemoniae*	CP006656.1	100%	99%
21.	B.8.2.1	*Klebsiella pnuemoniae*	CP013322.1	100%	99%
22.	B.8.3.1	*Enterococcus faecalis*	CP004081.1	100%	99%
23.	B.8.3.2	*Enterococcus faecalis*	CP004081.1	100%	99%
24.	B.8.3.3A	*Bacillus thuringiensies*	CP010088.1	100%	97%
25.	B.9A	*Klebsiella pnuemoniae*	CP006656.1	100%	99%
26.	B.9B	*Klebsiella pnuemoniae*	CP006656.1	100%	99%
27.	B.10.1	*Enterococcus faecalis*	CP004081.1	100%	100%
28.	B.11A	*Enterococcus durans*	KJ725230.1	100%	100%
29.	B.11B	*Bacillus antrachis*	CP008853.1	100%	100%

Furthermore, the preparation of phylogenetic trees of lactic acid bacterial isolates was made to determine the relative connection of the species based on genetic resemblance and the difference. The phylogenetic tree analysis results are shown in Figure 3.

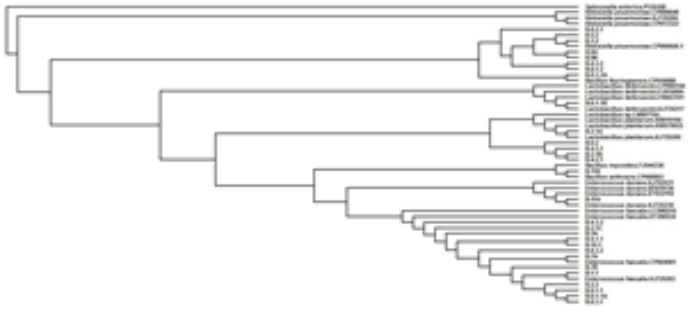
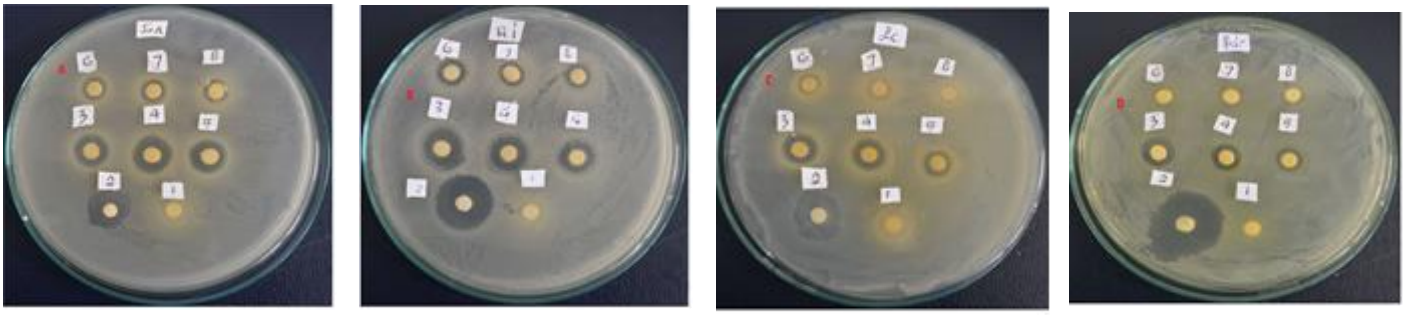


Figure 3. The phylogenetic tree of the lactic acid bacteria isolate origin of dadih is aligned with Genbank isolates

Antimicrobial activity of lactic acid bacteria against pathogenic bacteria

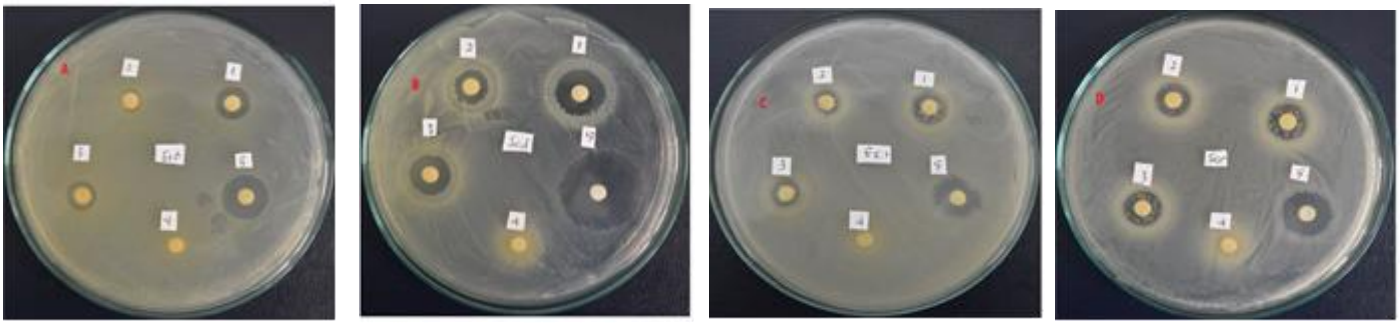
The Kirby-Bauer disc diffusion assay was carried out based on recommendations given the Clinical Laboratory Standards Institute. The results are presented in Figure 4 and table 4.



(a) (b) (c) (d)

Figure 4. Antimicrobial activity of Lactobacillus isolate against pathogen bacteria 4a. Salmonella enteritidis strain ATCC BAA-711; 4b. Salmonella enteritidis local Malaysia (code number 4301/15); 4c. Escherhicia coli strain ATCC 3540; 4d. Staphylococcus aureus strain ATCC 2592; 1. Negative control; 2. Positive control; 3. Lactobacillus plantarum 1; 4. Lactobacillus plantarum 2; 5. Lactobacillus plantarum 3; 6. Lactobacillus plantarum 4; 7. Lactobacillus plantarum 5 and 8. Lactobacillus delbrueckii.

Then, two Lactobacillus plantarum isolates were selected (Lactobacillus plantarum 1 and Lactobacillus plantarum 2) and L. delbrueckii for further testing of antimicrobial activity, the results are presented in Figure 5 and Table 5.



(a) (b) (c) (d)

Figure 5. Antimicrobial activity of Lactobacillus isolate against pathogen bacteria 5a. Salmonella enteritidis strain ATCC BAA-711; 5b. Salmonella enteritidis local Malaysia (code number 4301/15); 5c. Escherhicia coli strain ATCC 3540; 5d. Staphylococcus aureus strain ATCC 2592; 1. Lactobacillus plantarum 1; 2. Lactobacillus plantarum 2; 3. Lactobacillus delbrueckii; 4. Negative control and 5. Positive control.

Table 5. Inhibition zone of lactic acid bacteria origin dadih against pathogen bacteria

No.	Isolate	Inhibition zone against (mm)			
		S. enteritidis strain ATCC BAA-711	*S. enteritidis* local Malaysia	*E. coli* strain ATCC 3540	*S. aureus* strain ATCC 2592
1.	*L. plantarum 1*	12.0	16.0	11.0	12.0
2.	*L. plantarum 2*	8.5	12.0	9.0	10.0
3.	*L. delbrueckii*	10.0	14.0	10.0	11.5
4.	Negative control	6.0	6.0	6.0	6.0
5.	Positive control	15.0	21.0	13.0	13.0

IV. DISCUSSION

Isolation and identification of lactic acid bacteria

Twenty-nine colonies obtained from the isolation, selection and identification of the Kerinci dadih, Jambi have different characteristics. Overall colonies are milky white or creamy, circular shape, edge entire and convex elevation (Figure 1). Based on the size of the colony can be distinguished from 3 types, consisting of 17 small isolates, 6 medium sized and 6 large sized isolates (Table 1). Hayakawa (1992), reported the colony characteristic of lactic acid bacteria that grew on the media agar plates were different, the color of the colony depends on the origin of the isolate. Lactobacillus sp. colonies comes from milk is usually yellow, gray to brown around the colony, flat shape to bulge and sometimes irregularly colored. Brown color in the colony is due to the production of acids produced by the isolate of lactic acid bacteria. Some researchers report the characteristics of lactic acid bacteria colonies, both from fermentation milk and milk. Sunaryanto and Marwoto (2013) reported that colonies of lactic acid bacteria from dadih had a rounded and milky white color. Purwati et al., (2014) reported the colony of lactic acid bacteria from the Dadih Air Dingin, district Solok that was grown in medium MRSagar a milky white color, round shape with slippery edges and convex elevation. Nur et al. (2015) reports the morphology of lactic acid bacteria colonies from dangke having white or milk color, round shape with flat edge, the sparkling surface of the colony and form a clear zone around the colony and have different sizes (small to medium and medium to large).

Syukur et al. (2015) reported the morphology of colonies of lactic acid bacteria from dadih grown in solid medium having white or milky white, small size with smooth surface and surrounding organized colonies. Syukur et al. (2016) adds that colonies of lactic acid bacteria from dadih are rounded with soft and smooth surfaces. Maged et al. (2018) reported the morphology of lactic acid bacterial colonies grown on the MRS agar, having varying colors ranging from white to pale cream, has a circular shape and its size ranges from 0.5 to 4 mm.

In addition, colony morphology of lactic acid bacteria from buffalo milk was reported by Rizqiati et al. (2015) and Rizqiati et al. (2016) that the colony had an oval or round shape, white color and the formation of a yellow zone around the colony.

Morphological characteristic for LAB identification

Overall isolate lactic acid bacteria from dadih kerinci included in the genus Lacobacillus, because it appears as Gram-positive bacteria and does not have spores (Figure 2a and b). Based on the observed cell size, differentiated from 2 types consisted of 7 isolates in the form of bacilli, short, small and 22 isolates in the form of bacilli, length and large (Table 2). The morphological characteristics of lactic acid bacteria cells from fermented dairy products were reported by some researchers. Shi et al. (2012), reports that isolates of lactic acid bacteria from traditional fermented mare milk are bacillary and include Gram-positive bacteria. Sunaryanto and Marwoto (2013) report isolates of lactic acid bacteria from dadih include in Gram-positive bacteria with cellular bacillary shape and no spore. Purwati et al. (2014), reported that 11 isolates of lactic acid bacteria from the dadih Air Dingin Solok had a bacillary form and included Gram-positive bacteria. Syukur et al, (2014a) and Syukur et al. (2016) report that isolate lactic acid bacteria from the dadih is included in Gram-positive bacteria. Syukur et al. (2015) reports that the isolate of lactic acid bacteria of the dadih is included in Gram-positive bacteria with cellular coccus shaped cells in pairs or in chains. Nur et al. (2015) reports the isolate of lactic acid bacteria from dangke are bacillary shape and include Gram-positive bacteria. Dekumpitiya et al. (2016) reports that the isolate of lactic acid bacteria from Sri Lankan buffalo Milk Curd consisting of 69% were Lactobacillus sp and 21% were Streptococcus spp. Lactobacillus sp in curd is a Gram-positive bacterium with catalase is negative and Streptococcus spp in curd includes Gram-positive bacteria, oval-shaped, no spora, no motile and negative catalase. Maged S. Bin Masalam et al., (2018) reported isolate of lactic acid bacteria from raw and fermented milk (68%) included in Gram-positive bacteria.

Axelsson, (2004) reports that lactic acid bacteria are Gram-positive bacteria, bacilli shape or rounded, no spora, able to ferment carbohydrates, negative catalase and microaerophilic. The morphological characteristic of the lactic acid bacterial cells of the milk was reported by Khedid et al. (2009) that lactic acid bacteria from raw camel milk were included in Gram-positive bacteria, consisting of bacilli (37.5%) and coccus (62.5%). Setyawardani et al. (2011) reported that 33 isolates of lactic acid bacteria from Ettawa crossbreed and Saanen crossbreed were Gram-positive bacteria consisting of 26 isolates of rod shape, 1 isolate of short rod shape, 3 isolates of round, oval shape and 3 isolate of round shape. Mithun et al. (2015) reports that lactic acid bacteria from raw milk Aarey, India have rod- shaped, non spora forming and include Gram-positive bacteria. Rizqiati et al. (2015) reports that 84 of the 96 isolates of the original lactic acid bacteria from North Sumatera river buffalo milk include Gram-positive bacteria, consisting of 19 isolated of round shaped and 65of isolates rod-shaped. Rizqiati et al. (2016) reported that 21 of the 30 isolates of lactic acid bacteria from Pampangan buffalo milk, South Sumatra Indonesia include Gram-positive bacteria, consisting of 15 isolates of rod shaped and 6 of isolates round-shaped**.**

Identification of Lactobacillus isolates using 16S rRNA, sequencing and analysis

The phylogenetic approach is the latest system of bacterial taxonomies. The relationship between bacteria is known by comparing the molecular sequences base of primarily 16S rRNA gene. In the determination of species against lactic acid bacteria isolates from dadih originated Kerinci (Table 5) based on identification of carbohydrate fermentation test showed different results with molecular identification based on 16S rRNA base sequence analysis (Table 6). The result of identification with carbohydrate fermentation test was less accurate and requires confirmation of molecular identification to obtain the accuracy of lactic acid bacterial species identity quickly and accurately. This is in conformity with the opinion of Conter et al., (2005), which reports that identification of carbohydrate fermentation tests is less accurate and there are still errors in the determination of lactic acid bacterial species identity.

Based on the analysis results using BLAST (Table 6) and the preparation of phylogenetic trees (Figure 3), then from 29 isolates bacteria of the dadih original Kerinci obtained 5 isolates of Lactobacillus plantarum, 1 isolate of Lactobacillus delbrueckii, 13 isolates of Enterococcus faecalis, 1 isolate of Enterococcus durans, 7 isolates of Klebsiella pneumonia, 1 isolate of Bacillus thuringiensies and 1 isolate of Bacillus antrachis. Lactobacillus plantarum consists of B.2.1A, B.2.1B, B.4.2.1, B.4.3.1, B.6.2 and 1 isolate of Lactobacillus delbrueckii that is B.6.1.1B.

Isolate B2.1A has a close relationship with Lactobacillus plantarum KJ725205.1 (100% query coverage and 100% identification maximum), isolatesB2.1B, B4.3.1 and B6.2had a close relationship withLactobacillus plantarum KR816164.1 (100% query coverage and 100% identification maximum) and isolates B4.2.1 have a close relationship with Lactobacillus plantarum KR816164.1 (100% query coverage and 99% identification maximum). Furthermore, isolates B6.1.1B have a close relationship with Lactobacillus delbrueckii KJ725217.1 (100% query coverage and 100% identification maximum).

Some researchers reported the identification results of lactic acid bacteria molecularly in fermented milk. Shi et al. (2012) reported that from traditional fermented mare milk obtained 25 isolatess of Lactobacillus rhamnosus and 2 isolates of Lactobacillus fermentum. Sunaryanto and Marwoto (2013), reported that Lactobacillus plantarum is a dominant lactic acid bacterium isolated from dadih Payakumbuh, West Sumatera. Purwati et al. (2014) reported that Lactobacillus plantarum is a lactic acid bacterium found in dadih that comes from Air Dingin, Solok district West Sumatera. Furthermore, Syukur et al (2014a) reported that Lactobacillus plantarum is a lactic acid bacterium found in dadih that comes from Pematang Panjang, Sijunjung district West Sumatera**.**

Antimicrobial activity of lactic acid bacteria against pathogenic bacteria

The inhibition strength of microbial activity in inhibiting the growth of pathogenic bacteria is influenced by microbial species. Different species will result in different inhibitions and activities due to differences in the resulting metabolite components. Strength rating of

inhibitory activity against isolates of Gram-negative bacteria Salmonella enteritidis local Malaysia (code number 4301/15), Salmonella enteretidis strain ATCC BAA-711 and Escherichia coli strain ATCC 35401 is Lactobacillus plantarum 1 and Lactobacillus plantarum 2> Lactobacillus plantarum 3> Lactobacillus plantarum 5 > Lactobacillus plantarum 4 > Lactobacillus delbrueckii. While the strength of the inhibitory activity against isolates of Gram-positive bacteria Staphylococcus aureus strain ATCC 25923 is Lactobacillus plantarum 1 and Lactobacillus plantarum 2 > Lactobacillus plantarum 3> Lactobacillus delbrueckii > Lactobacillus plantarum 4 and Lactobacillus plantarum 5 (Figure 4 and Table 4).

Based on testing of advanced antimicrobial activity, it is obtained that the rank strenght of inhibition activity againts isolates of Gram-negative bacteria Salmonella enteritidis local Malaysia (code number 4301/15), Salmonella enteretidis strain ATCC BAA-711 and Escherichia coli strain ATCC 35401 is Lactobacillus plantarum 1> Lactobacillus delbrueckii > Lactobacillus plantarum 2. While the strength of the inhibitory activity against isolates of Gram-positive bacteria Staphylococcus aureus strain ATCC 25923 is Lactobacillus plantarum 1> Lactobacillus delbrueckii > Lactobacillus plantarum 2 (Figure 5 and Table 5).

Lactobacillus plantarum and Lactobacillus delbrueckii show inhibition activity against isolates of Gram-negative bacteria (Salmonella enteritidis local Malaysia code number 4301/15, Salmonella enteretidis strain ATCC BAA-711 and Escherichia coli strain ATCC 35401) as well as Gram-positive bacteria positif (Staphylococcus aureus strain ATCC 25923). Overall, Lactobacillus plantarum showed good antimicrobial activity against Gram-negative bacteria, especially Salmonella enteritidis local Malaysia (code number 4301/15) and Salmonella enteritidis strain ATCC BAA-711 compared with a positive control using amoxicillin antibiotics. However Lactobacillus plantarum 1 including the bacteria that possess a strong inhibitory activity, the case according according to the opinion of Jacobsen et al., (1999) which states bacteria including strong inhibitory activity category when zone of inhibition greater than 5 mm.

The inhibition strength of Lactobacillus plantarum inhibits the growth of pathogenic bacteria caused by the resulting metabolite component. Lactobacillus plantarum is a homofermentative type lactic acid bacteria, is anaerobic facultative and this bacterium is able to ferment lactose and produce 85% lactic acid. The lactic acid produced causes a decrease in the pH of the environment to grow to 4.0 lower than the optimum pH of pathogenic bacteria that is about 6.5-7.5. Low environmental pH conditions can inhibit bacterial growth (Surono, 2004). Marianelli et al. 2010 reported that this metabolite component was administered by bacteria during the initial phase of their life cycle.

The effectiveness of inhibition of Lactobacillus plantarum against pathogenic bacteria may be activating, damaging or destroying cells that progress towards death. The inhibition effects of pathogen bacteria Escherichia coli, Salmonella typhimurium and Staphylococcus aureus are largely due to the accumulation of organic acids produced. Acid will cause a decrease in pH below the pH range of bacterial growth, wherein this acid is in a non-dissociated form that can rapidly diffuse into microbial cells. According to Osling and Lindgren (1990), non-dissociated acids will decompose into anions and protons, in which protons (H+) will enter the cell, resulting in metabolic function disrupted such as cytoplasmic acidification, substrate transfer inhibition, macromolecular synthesis, which overall bacterial growth will be impeded. Some researchers report the bacterial ability of lactic acid bacteria in inhibiting pathogenic bacterial growth activity. O'Sullivan et al. (2002) and Akpinar et al. (2011) reported that Lactobacillus lactis and Streptococcus thermophilus had the ability to inhibit the process of decomposition of food and growth of pathogenic bacteria as well as maintaining the nutritional quality of raw materials for longer shelf life. Srinivasan et al. (2012) reports that Lactobacillus plantarum isolated from soy milk also has a strong antibacterial activity against Escherichia coli and other pathogenic bacteria. Carbon dioxide which is a bio-fermentation product of other lactic acid bacteria can cause dysfunction in lipid bilayer permeability. It can also inhibit the growth of many microbial of food decay, especially psychotrophic Gram-negative bacteria. Diacetyl is suspected to react with arginine-binding protein from Gram-negative bacteria and therefore disrupts the use of arginine. Sunaryanto and Marwoto (2013) report that Lactobacillus plantarum of buffalo milk dadih has the strongest inhibitory activity of Staphylococcus aureus, followed by Enterococcus faecalis and Escherichia coli. Syukur et al. (2014a) reported that Lactobacillus plantarum isolated from the dadih from Sijunjung, West Sumatra has a good inhibitory to pathogenic bacteria. The inhibition zone for pathogenic bacteria Staphylococcus aureus is greater than Escherichia coli and Salmonella thypii. Syukur et al. (2015) reports that the lactic acid bacteria isolated from the dadih from Lareh Sago Halaban Payakumbuh, West Sumatra the most powerful antimicrobial activity is Staphylococcus aureus followed by Escherichia coli and Salmonella thypii. Syukur et al. (2016) reported that lactic acid bacteria isolated from the dadih of Payakumbuh, West Sumatra had the strongest antimicrobial activity followed by lactic acid bacteria from dadih from Solok and Sijunjung, and generally the inhibition zone for Gram-positive bacteria was greater than Gram-negative bacteria**.**

Conclusions

Twenty-nine bacterial colonies originating from fermented milk buffalo (dadih) successfully isolated, identified and characterized. Identification by using molecular analysis PCR (genotypic characterization) has detected 5 isolates of Lactobacillus plantarum, 1 isolate of Lactobacillus delbrueckii, 13 isolates of Enterococcus faecalis, 1 isolate of Enterococcus durans, 7 isolates of Klebsiella pneumonia, 1 isolate of Bacillus thuringiensies and isolate of Bacillus antrachis. Lactobacillus plantarum 1 is included in the bacterial category showing strong inhibition activity, particularly inhibiting Salmonella enteritidis local Malaysia code number 4301/15 and Salmonella enteritidis strain ATCC BAA-711.

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