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Characterization of lactic acid bacteria from buffalo dairy product (*dadiah*) as potential probiotics

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Abstract. *Aritonang SN, Roza E, Yetmaneli, Sandra A, Rizqan. 2022. Characterization of Lactic Acid Bacteria from Buffalo Dairy Product (*Dadiah*) as Potential Probiotics. Biodiversitas 23: 4418-4423. Dadiah* is one of fermented milk from West Sumatra which made from buffalo milk. These product made traditionally by pouring fresh buffalo milk into a bamboo stick covered with banana leaves and fermenting at room temperature for two days. The study's objective was to isolate and identify Lactic Acid Bacteria (LAB) from *dadiah* as candidates for probiotics. The factors that are considered are LAB Characterization, Biochemical and Microscopic Characterization of LAB Isolate, Bacterial Resistance in Acidic conditions, Bile Salts Resistance, and Antimicrobial Activity. Furthermore the isolate identify by Polymerase Chain Reaction (PCR) using 16S rRNA. Samples of *dadiah* were collected from five farmers in Tanjung Bonai Village, North Lintau Buo Regency, Tanah Datar District, West Sumatra, Indonesia. Observations were made in four replications, and the data obtained were analyzed descriptively. The total colony of lactic acid bacteria from *dadiah* was $7x10^8$-$23x10^8$ CFU/mL. LAB isolates from *dadiah* were classified as Gram-positive bacilli. The isolates are resistant to acidic conditions and bile salts and have inhibitory activity against pathogenic bacteria. Identifying the selected isolate using the 16S rRNA showed that the isolate had similarities with *Lactobacillus casei* strain HDS-01. It can be concluded that *dadiah* used in this study is as a probiotic food.

Keywords: 16S rRNA, *Dadiah*, LAB, *Lactobacillus casei* strain HDS-01, probiotics

INTRODUCTION

Buffalo had existed in West Sumatra for several centuries ago. Buffalo has an essential function in people's lives and is a symbol of the traditional culture of the West Sumatra region. Almost all buffalo in West Sumatra are swamp buffalo. It's generally bred and raised in rural areas to plow rice fields and as meat producer. However, in lactation period the milk produced is also used by farmers to make fermented milk. Buffalo's milk production ranges from 1.5 to 2 L of milk/day (Purwati et al. 2016). Fermented milk products are one aspect of biotechnology that utilizes beneficial bacteria for health.

In West Sumatra, buffalo milk is processed into a traditional food known as *dadiah*. The process of making *dadiah* is by pouring fresh buffalo milk into a bamboo stick covered with banana leaves and fermenting at room temperature for two days. During the fermentation process, there will be a decrease in pH, and clumping will occur in the milk so that it is formed like milk tofu, white in color, soft in texture, and has a specific aroma. The fermentation process in *dadiah* occurs due to the presence of microbes that occur naturally in bamboo tubes. The main microbes that play a role in this fermentation process are lactic acid bacteria (LAB). LAB is a group of bacteria capable of converting carbohydrates (lactose) into lactic acid (Purwati et al. 2016). During the fermentation process, LAB produces organic acids, lowering the pH of the environment to 3 - 4.5. LAB has long been used in food fermentation processes to preserve and improve the texture and taste of foodstuffs. In addition, LAB produces natural preservatives such as hydrogen peroxide, diacetyl, carbon dioxide, acetaldehyde, amino acid and bacteriocin that can inhibit the growth of pathogenic bacteria (Smid and Gorris 2007). The bacteriocin in LAB positively impacts human health (Jagadesswari 2010).

With the content of lactic acid bacteria in *dadiah*, this has potential as a probiotic. Because in general, bacteria that have a probiotic function are classified as lactic acid bacteria. Lately, research on probiotics has been very much done. This is because probiotics have myriad benefits for humans who consume them. Probiotics are foods or supplements that contain live microbes that positively affect health when consumed in specific amounts (Handayani et al. 2016). Probiotics produce enzymes that can break down complex molecules such as long-chain carbohydrates, proteins, and fats into simpler molecules (Okfriani et al. 2018), thus helping absorb nutrients. Probiotics have therapeutic benefits such as assisting in treating lactose intolerance, preventing colon cancer, and lowering cholesterol levels in the blood. According to Salminen et al. (2004), The conditions that must be met by lactic acid bacteria that function as probiotics include; are not pathogenic microbes that can represent typical intestinal microorganisms from specific hosts and are still active under conditions of gastric acid and high concentrations of bile salts in the small intestine; good probiotics must be able to grow and metabolize quickly and be present in high amounts in the gut; ideal probiotics can temporarily colonize some parts of the intestine; probiotics

can produce organic acids efficiently and have antimicrobial properties against harmful bacteria; easy to produce, capable of growing in large-scale production systems and viable during storage conditions.

One producing area of *dadiah* is the Tanjung Bonai Village, North Lintau Buo District in Tanah Datar Regency. This area is one of the *dadiah* centers' production due to its high buffalo population. According to the Central Bureau of Statistics (2016), buffalo milk production in Tanah Datar Regency is 20,927 liters/year. High milk production encourages most farmers to produce *dadiah*. Therefore, lactic acid bacteria (LAB) in *dadiah* have great health potential and could be used as a natural preservative in the food industry. This study aims to obtain the best probiotics from *dadiah* that give more benefits to the community.

MATERIALS AND METHODS

Sample collection

Dadiah samples were obtained from five farmers in Tanjung Bonai Village, North Lintau Buo Regency, Tanah Datar District, West Sumatra, Indonesia. LAB Isolation and Characterization, Biochemical and Microscopic Characterization of LAB Isolate, Bacterial Resistance in Acidic conditions, Bile Salts Resistance, and Antimicrobial Activity were carried out in the Livestock Products Technology. Laboratory. Meanwhile, identification of LAB using 16S rRNA was carried out at the Laboratory of Animal Products Technology, Faculty of Animal Husbandry, Andalas University, Indonesia.

Isolation and purification of lactic acid bacteria (LAB)

The LAB was isolated and purified according to the method described by Purwati et al. (2005). First, one g of the *dadiah* sample was diluted with 9 mL MRS broth. Next, a 10^{-1} dilution was placed into an anaerobic jar and incubated for 24 h at 37ºC. The 10^{-1} dilution was then placed into a test tube containing 0,9 mL MRS broth solution and serially diluted in 10^{-6}. Next, 100 uL samples of the 10^{-6} dilution were spread on a Petri dish containing MRS agar and incubated at 37ºC for 48 h. After 48 hours of incubation, the single colony of lactic acid bacteria, with a smooth, round yellowish-white color, was transferred to MRS Agar media (MERCK) for colony purification by streak method using an ose needle and then incubated for 24 hours at 37°C.

Isolate characterization

Gram stain and catalase reaction tests were conducted to determine the isolates. The morphology of the isolated cells was evaluated microscopically. The LAB gas production from glucose was tested using a Durham tube using MRS broth to determine the type of fermentation (Romadhon et al. 2012).

Acid resistance test

LAB culture (1 mL) was inoculated on 9 mL of MRS Broth and incubated at 37ºC for 24 hours. A total of 1 mL of bacterial culture from MRS Broth was put into a test tube containing 9 mL of MRS Broth without adding HCl (control). Another 1 mL of bacterial culture from MRS Broth was put into a test tube containing 9 mL of MRS Broth at pH 3 (pH was adjusted by adding 5N HCl), then incubated for 90 minutes, followed by diluting to 10^{-6}. The dilution culture was inoculated at MRS Agar using the spread method and incubated at 37ºC for 48 hours. The colony-Forming Unit calculates bacteria that can survive (CFU). The comparison of the number of cells before and after incubation was expressed as viability (%). The higher the viability percentage indicates that the bacteria are more resistant to low pH (Sunaryanto and Marwoto 2013).

Bile salts resistance test

One mL of LAB culture was inoculated on 9 mL MRS Broth media and incubated at 37ºC for 4 hours. Then, as much as 1 mL of bacterial culture from MRS Broth was put into a reaction tube containing 9 mL MRS Broth without ox gall (control), and the other was put on MRS Broth with 0.3% ox gall incubated overnight at 37ºC. Next, the culture in ox gall 0.3% and control culture was diluted to 10^{-6} and then cultivated using the spread method on the MRS media, then incubated at 37ºC for 48 hours. The Colony Forming Unit calculated the number of surviving bacteria (CFU). Comparing the cell numbers before and after incubation was expressed as viability (%). The higher the viability percentage indicates, the more bacteria resistance to bile salts (Sunaryanto dan Marwoto 2013).

Antimicrobial activity

Antimicrobial activity was performed using the agar well diffusion method against three bacteria: *Escherichia coli* O157, *Listeria monocytogenes* CFSAN 004330, and *Staphylococcus aureus* ATCC 25923. First, 1 mL of LAB culture was centrifuged at 10.000 rpm for 5 minutes at 27ºC. The supernatant cell-free was used as a microbial resistance. Furthermore, 20 mL of sodium agar (NA) medium was added with 0.2% of pathogenic bacteria and allowed to cool in a Petri dish. Next, wells with a diameter of ±6.5 mm were made on the NA media. After that, antibiotics such as penicillin, kanamycin, and ampicillin were added to paper disks as positive controls. Finally, the inhibition zone formed on the paper disk was compared with the inhibition zone formed during the antimicrobial activity testing against pathogenic bacteria (Yang et al. 2012).

Identification of LAB using 16S rRNA

The isolation of DNA was carried out using KIT Promega (USA). LAB isolates from MRS Broth were pipetted (1000 µL), included in the new Eppendorf, and centrifuged at 14000 rpm for 2 minutes. The supernatant was removed, and the pellet was added with 480 µL 50 mM EDTA. Then, 120 µL of Lysozyme was added, followed by incubation at 37ºC in a water bath for 60 minutes. And then, centrifuge for 2 minutes at 14000 rpm. Remove the supernatant, and the pellet was taken and added with 600 µL nuclei lysis solution, then incubated at 80ºC for 5 minutes. After incubation, let it stand at room

temperature and, followed by adding 3 µL of RNase solution, set it in a water bath at 37ºC for 60 minutes. The next step was the addition of 200 µL of protein precipitation solution and then vortex. Finally, 600 µL of isopropanol was added. Centrifuged for 2 minutes at 14000 rpm. Pellets were taken, and the supernatant was removed.

Furthermore, 600 µL of ethanol 70% was added to the pellets and then homogenized. Centrifuged for 2 minutes at 14000 rpm, and the pellets were taken. DNA pellets were rehydrated by adding 10-100 µL of Rehydration solution for 30 minutes at 65ºC. Initial denaturation was carried out at 95°C for 5 minutes with 25 cycles, followed by PCR denaturation at 95°C for 45 seconds and annealing at 56°C for 45 seconds. The extension was carried out at 72°C for 1 minute 40 seconds, and the final extension was at 72°C for 10 minutes. The resulting DNA was separated using electrophoresis at 100 V for 21 min, using 1% agarose in 1x TAE buffer. Then, a gel was observed under the UV lamp. The 16S rRNA gene sequences of the isolate were submitted to the NCBI for a BLAST search. The MEGA version 7.0 (http://www.megasoftware.net) created phylogenetic trees using the neighbor-joining (NJ) method.

RESULT AND DISCUSSION

LAB isolation characterization

The total LAB colony from *dadiah* collected from five different farmers in Lintau Buo Utara Regency Tanah Datar District is presented in Table 1.

The total LAB colonies of *dadiah* from five farmers ranged from 7 x10^8 CFU/mL to 23 x10^8 CFU/mL. Meanwhile, the minimum number of probiotic bacteria in food should be 10^6 CFU/g, or the number of probiotics consumed daily is equivalent to about 10^8 CFU/g (Shah 2007).

In this study, the LAB colonies of *dadiah* were higher than *dadiah* from Sijunjung Regency (1.48 x 10^8 CFU/g (Purwati et al. 2016). Total LAB colonies of *dadiah* are influenced by the quality of the fresh milk and nutrient content in the substrate. The nutrient influenced the growth of microorganisms (Moat et al. 2002). Basic nutrients such as carbon, nitrogen, energy, minerals, and vitamins are required to support growth. The natural fermentation process in *dadiah* involves microbes on the inner surface of the bamboo tube, the surface of the leaf cover, and the buffalo milk (Usmiati and Risfaheri 2012).

Macroscopically, all single colonies of LAB had a white-yellowish color with a round shape, flat and smooth edges, and smooth and slightly combed elevation. However, Syukur and Purwati's (2013) study showed that single colonies of lactic acid bacteria have a round shape, cream color, and smooth edges.

Biochemical and microscopic characterization of LAB isolate

The Gram staining test showed that the LAB obtained in this study were Gram-positive (+) which absorbed purple dye from crystal violet. Some are round, and some are rod-shaped (Table 2).

LAB is a facultative anaerobic bacterium, Gram-positive, rod-shaped or round, does not produce spores, and ferments carbohydrates to produce lactic acid (Salminen et al. 2004). The characteristics of lactic acid bacteria are compact and thick peptidoglycan (Fevria 2019). Gram-positive bacteria can retain crystal violet dye during the Gram staining process. All LAB isolates produced gas. LAB is a homofermentative bacteria with lactic acid as the main product. Homofermentative bacteria is indicated by the absence of gas bubbles in the Durham tube, which is placed in the MRS Broth (MERCK®) media. It converts glucose into lactic acid through the glycolysis pathway and does not produce CO_2 (Syukur and Purwati 2013). Bacteria belonging to homofermentative groups include *Leuconostoc*, *Weissella*, and some *Lactobacillus* (Ross et al. 2002).

The catalase test results showed that all isolates from *dadiah* had negative catalase, as indicated by the absence of air bubbles or O2 gas. It is because catalase breaks down hydrogen peroxide into water and oxygen gas. On the contrary, a positive catalase reaction is indicated by the formation of air bubbles or oxygen production (Syukur et al. 2014).

Table 1. Total of Lactic Acid Bacteria colonies from five farmers

Sample code	Total colony of Lactic Acid Bacteria (x10^8 CFU/g)
A	12
B	7
C	23
D	13
E	15

Table 2. Characteristics of Lactic Acid Bacteria from *dadiah*

Sample	Gram staining	Catalase test	Cell shape	Gas production test
A	+	-	Rod	+
B	+	-	Rod	+
C	+	-	Round	+
D	+	-	Round	+
E	+	-	Rod	+

Bacterial resistance in acidic conditions

The resistance test of curd from Lintau Buo Utara against acidic conditions showed that the number of living bacteria (without a pH adjustment) was 8.3-12.5 x107 CFU/g. However, after three pH settings, the number of living bacteria ranged from 7.8 - 12.3 x107 CFU/g. So, the viability of lactic acid bacteria ranged from 68.45-98.00%, with the highest isolate of lactic acid bacteria being sample C (98%). One of the characteristics of probiotics is that they must be resistant and able to live in acidic conditions. The higher the viability of LAB, the higher the resistance of LAB isolates under acidic conditions. The decrease in total LAB colonies at pH 3 could be caused by bacterial death due to cell wall damage in very acidic conditions. According to Susanti et al. (2007), Most lactic acid bacteria grow more slowly at low pH, which causes decreased cell viability, but it depends on bacterial strain (Susanti et al. 2007a). Exposure to highly acidic conditions can cause membrane damage and the loss of intracellular components such as Mg, K, and fat from cells, leading to the death of bacteria that are not resistant to acid.

Lactic Acid Bacteria (LAB) isolated from *dadiah* were considered probiotic bacteria because they can survive in the digestive tract. Harun et al. (2020) stated that the higher the viability of LAB, the higher the resistance of lactic acid bacteria in the digestive tract.

Bile salts resistance

The results of the bile salts resistance test showed that the total LAB in the 0.3% bile salts treatment for 4 hours was 0.43 -0.60 x 10^8 CFU/mL, while the total LAB in control (without the addition of 0.3% ox gall) was 0.87 - 1.02 x 10^8 CFU/mL. Sample C produced the highest viability by 59%. Viability is the ratio of living cells before and after incubation at 0.3-0.5 % bile salt concentration and was expressed in percentage (%). The higher the viability of LAB produced, the higher the resistance of LAB bacteria to bile salts. The LAB isolates from sample C of *dadiah* had better bile salt resistance than *Lactobacillus fermentum* strain IMAU70167 isolated from buffalo milk with viability ranging from 32.23-56.13%, in 0.3% bile salts for 4 hours (Melia et al. 2018).

LAB resistance to bile salts is one of the important characteristics of LAB because it affects the LAB activity in the digestive tract, especially in the upper intestinal tract, where bile salts are secreted. Gall has properties as a surface-active compound by activating the lipolytic enzymes secreted by the pancreas. This lipolytic enzyme reacts with fatty acids in the bacterial cytoplasmic membrane, causing changes in membrane structure and permeability properties that affect resistance to bile salts. The resistance of lactic acid bacteria towards bile salts is related to the Bile Salt Hydrolase (BSH) enzyme, which helps to hydrolyze conjugated bile salts, thereby reducing the toxic effects on cells.

Antimicrobial activity

Antimicrobial activity was conducted on sample C of *dadiah* due to its high resistance to low pH and bile salt (Table 5).

The results showed that LAB from sample C had better inhibitory activity than penicillin, ampicillin, and kanamycin antibiotics against *Escherichia coli*. Ampicillin has greater inhibitory activity than LAB isolates from sample C, penicillin, and kanamycin against *Staphylococcus aureus* and *Listeria monocytogenes*. The clear zone indicated inhibition of bacterial growth. The larger the clear zone, the greater the inhibition of bacterial pathogenic growth (Pelczar and Chan 2008). Morales et al. (2003) categorized the inhibition zone as follows: weak activity (<5mm), moderate (5-10mm), strong (>10-20mm), and very strong (>20-30mm). Based on these categories, LAB isolates from Sample C of *dadiah* had very strong antibacterial activity against *Escherichia coli* 0157 and *Staphylococcus aureus.*

Soleha (2015) stated that penicillin is an antibiotic that inhibits bacterial growth by inhibiting bacterial cell wall synthesis and belongs to the beta-lactam class. Penicillin actively inhibits Gram-positive bacteria. Ampicillin is a penicillin derivative that inhibits the growth of Gram-positive and Gram-negative bacteria and belongs to the beta-lactam group. Furthermore, kanamycin has bactericidal aminoglycosides, active against Gram-positive bacteria that cause infections.

Table 3. Viability percentage of bacteria isolated from *dadiah* in acidic conditions or low pH

Sample	Total colony (CFU/g)		Viability (%)
	pH Control	pH 3	
A	10 x 10^7	9.2 x 10^7	75,32
B	8.3 x 10^7	7.8 x 10^7	68.45
C	12.5 x 10^7	12.3 x 10^7	98.00
D	11.2 x 10^7	9.9 x 10^7	75.23
E	10.5 x 10^7	8.2 x 10^7	87.43

Table 4. Viability percentage of LAB in the 0.3% ox gall at pH 3

Sample	Total Coloni (CFU/g)		Viability (%)
	pH Control	pH 3	
A	1.00 x 10^8	0.58 x 10^8	45.67
B	0.87 x 10^8	0.47 x 10^8	47.78
C	1.02 x 10^8	0.60 x10^8	59.00
D	0.96 x 10^8	0.60 x 10^8	56.82
E	0.98 x 10^8	0.43 x 10^8	58.21

Table 5. Diameter of growth inhibition of LAB against 3 bacterial isolates

Inhibitory source	Clear zone diameter (mm)		
	***Escherichia coli* 0157**	***Staphylococcus aureus***	***Listeria monocytogenes***
LAB (C)	22.27	20.25	5.10
Penicillin	2.7	2.7	2.7
Ampicillin	15.20	24.29	18.23
Kanamycin	13.18	17.22	6.11

Identification of LAB using 16S rRNA

The sequencing results were used to identify molecular lactic acid bacteria from *dadiah* isolates by comparing homology with 16S rRNA gene sequences in GeneBank (www.ncbi.nlm.nih.gov) using the BLAST program. The PCR sequencing result of LAB from sample C of *dadiah* was presented in Figure 1. The phylogenetic tree of LAB from sample C of *dadiah* was presented in Figure 2.

Based on the results, LAB from sample C of *dadiah* had 99% similarity to *Lactobacillus casei* strain HDS-01. Hagstrom et al. (2000) stated that isolates with more than 97% similarity of 16S rRNA sequences were considered the same species. In contrast, 93-97% of sequence similarities were considered the same genus but different species.

The different types of bacteria in buffalo milk and *dadiah* from the same farmer were caused by the bamboo used to make *dadiah*. Purwati et al. (2010) stated that microbes in *dadiah* could be originated from banana leaves as a bamboo cover, the milk itself, and the bamboo tubes used that result in different types of bacteria. Furthermore (Purwandhani et al. 2018), stated that various LAB in *dadiah* fermentation varied due to differences in location and natural fermentation without the addition of a previous starter. Bacteria are commonly found in fermented foods such as yogurt, cheese, pickles, kimchi (Syukur and Purwati 2013), and okara (Aritonang 2017). *Lactobacillus fermentum* L23 has been isolated from buffalo milk from Agam Regency (Melia et al. 2018), while *L. fermentum* and *L. plantarum* have also been isolated from *dadiah* from Lintau and Air Dingin (Amelia et al. 2020; Harun et al. 2020). A study by Sukma (2018) showed that bacteria from *dadiah* were dominated by bacteria from the *Lactococcus*, *Lactobacillus*, and *Leuconostoc* groups, all of which are classified as LAB.

In conclusion, *dadiah* from five farmers in Nagari Tanjung Bonai, North Lintau Buo District, Tanah Datar Regency, contained LAB with the total colony ranging from 7 x 10^8 - 23 x 10^8 CFU/mL. The LAB isolated from *dadiah* was Gram-positive bacilli with the viability in 0.3% bile salts, 0.3% was 59%, and resistance to pH 3 with the viability of 98%. This isolate was most active in inhibiting the growth of *E. coli* O157 with an inhibition zone of 22.27 mm and identified as *Lactobacillus* casei strain HDS-01.

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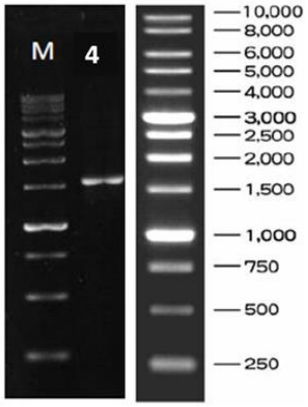

Figure 1. PCR amplification of ribosomal RNA using l1492R and 27F.4 is a lactic acid bacteria isolated from *dadiah* originated from Tanah Datar. Lane M = Marker; Lane 4: Total base pair (bp) in the *dadiah* sample

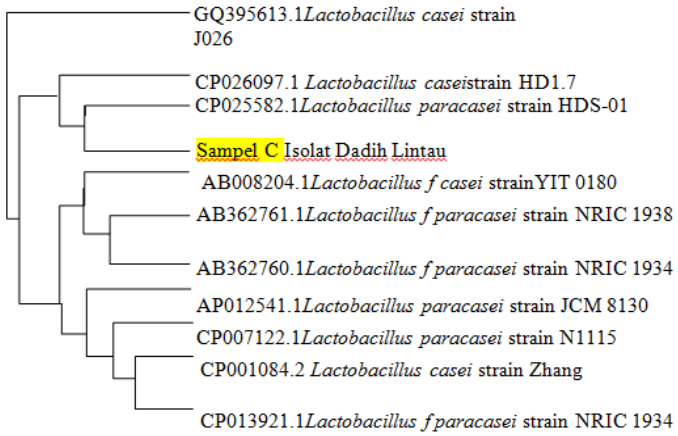

Figure 2. The phylogenetic tree of 16S rRNA of LAB from sample C of *dadiah* using the neighbor-joining method

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