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Article

Molecular identification of lactic acid bacteria an approach to sustainable food security

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Abstract

Background: Dadiah is a traditional dish from West Sumatra made from buffalo milk, which is fermented in bamboo tubes and left at room temperature for ±2 days. Dadiah is included in the staple food category because it contains Lactic Acid Bacteria (LAB), which has the potential to be a probiotic. This study aims to determine the identification and characterization of LAB from Dadiah from Halaban, Kab. Fifty Cities, West Sumatra.

Design and Methods: A survey method was used in this research with a descriptive analysis, Antimicrobial activity testing was done with bacteria *Escherichia coli O157*, *Staphylococcus aureus*, *Listeria monocytogenes*, and *Listeria innocua*. Molecular identification was done using the 16S rRNA gene.

Results: Probiotic candidate test with the best results in testing for resistance to stomach acid at pH3 with the viability of 65.98%, bile salt resistance 0.3%, viability of 54.90% from 2DA isolates. Antimicrobial activity with the best clear zone area results was obtained in 2DA isolates with *Escherichia coli* O157 test bacteria of 21.16 mm, *Staphylococcus aureus* with a clear zone area of 23.17 mm, *Listeria innocua* of 19.24 mm and *Listeria monocytogenes* with a clear zone area 18.23 mm in 4DA isolate, LAB identification using 16S sRNA gene, results of running PCR base length 1419bp.

Conclusions: Phylogenetic analysis shows that Dadiah of Limapuluh Kota Regency is a kin to *Lactobacillus plantarum*. The superiority of identification technology by using 16S rRNA gene only can be conducted if the nucleotide sequence information of the targeted bacteria is known beforehand.

Introduction

Dadiah is a fermented dairy product. *Dadiah* is simply made by pouring fresh buffalo milk into a bamboo tube which is then covered by banana leaf and let sit for two to three days in cool place. *Dadiah* is considered as probiotic dairy product category since it was product of fermented milk and contain lactic acid bacteria. *Dadiah* is one of the popular processed dairy products in West Sumatera, such as in Bukittinggi, Padang Panjang, Solok Limapuluh Kota, and Tanah Datar.[1] Currently, *Dadiah* is consumed as a traditional food, served at weddings and while giving an honorable title "Datuk" in West Sumatra.[2] About ±150mL freshly milked buffalo milk is poured into a piece of freshly cut section of bamboo and covered with banana leaf. It is then fermented naturally at room temperature by microbes present in the bamboo, the banana leaf, and the milk itself until it forms lump. After 24 hours, the milk will clot forming pudding or yellowish white tofu, which is condensed, and has distinct aroma (combination of milk and bamboo). After the fermentation, *dadiah* can be eaten directly.[3]

The existence of Lactic acid bacteria (LAB) occur naturally in food and it is used as safe fermentation process for a long period of time and also its advantage on health made it regarded as Generally Recognized as Safe (GRAS) microorganism to be consumed by human.[4] According to several previous researches, it is known that *dadiah* contain plenty of LAB which makes it very potential as probiotic.[1,5]

According to FAO and WHO (2017), in order to categorize LAB as probiotic, species characterization of the strain must be done in order to claim its functional characteristics.[4] Indigenous identification of LAB was originally done by phenotypic method including tests on cell morphology, physiology, biochemical test, and cell's ability in fermenting several substrates of carbohydrate or identification by using API test kit. However, the phenotypic method has several drawbacks, especially determining the species of bacteria. Molecular method approach such as nucleic acid analysis can be used to resolve this drawback. Identification through molecular method can give more accurate information on identification of LAB.[6]

LAB has advantageous effects on health by aiding the stabilization of the growth of intestinal micro flora, preventing the growth of pathogenic microbes, so that it can stimulate the increase of immune function and resistance to infection. Nowadays, LAB probiotic is an interesting research object in food industry and international research topic due to its functions as probiotic, such as producing antimicrobial compound,[7-12] decreasing serum cholesterol level[13] preventing "lactose intolerance", stabilizing intestinal micro flora (probiotic),[14] as anti-obesity,[15] as antioxidant activity and anti-infalmatory,[16,17] as antimutagen,[18,19] as anticancer,[20] as antitumor,[21] anti-aging for kidney,[11] and modulating brain function.[22] Fermentation process

Significance for public health

Molecular Identification (Phylogenetic analysis) of Lactic Acid Bacteria shows the Approach to Sustainable food Security United Nations Sustainable Goal 2 End hunger, achieve food security and improved nutrition and promote sustainable agriculture.

by using LAB will yield product with high nutrition and health-related function (probiotic), with unique taste.[23,24] LAB function on decreasing lactose intolerance, treating or preventing diarrhea and also controlling risk and infection on digestive tract,[14] stimulating immune system,[25,26] and suppressing the growth of intestinal pathogenic bacteria and also increasing the growth and colonization of advantageous intestinal microbes,[27] lowering the amount of dangerous enzymes in feces and controlling water content in feces,[28] as anti-obesity,[15] antioxidant,[29] anti-inflammatory,[15,16] antimutagen,[17,18] anticancer,[20,30] antitumor,[21] hypocholesterolemia,[13] as antihypertensive,[31-33] and modulating brain function.[23] This study aimed to understand the molecular identification of LAB present in *Dadiah* both conventionally and with molecular analysis, which has potential as probiotic.

Design and Methods

Sample of *dadiah* is obtained from Lareh Sago Halaban District, Limapuluh Kota

Regency, West Sumatera, Indonesia straight from the farmer, which is then brought to Animal Product Technology Laboratory of Faculty of Animal Science of Anadalas University in order to analyze.

Isolation and identification of lactic acid bacteria

Macroscopic identification

Media *de Mann Ragosa Sharpe* (MRS) broth was used for dilution. BAL was spread with methods spread, at inoculation and stored in anaerobic jar after with incubation for 48 hours at a temperature of 37°C. Single colony that characterize BAL is round, smooth and white yellowish in color were then transferred to media de Mann ROGOSA Sharpe MRS for purification of colony with streak methods and incubated for 24 hours at a temperature of 37°C.

Microscopic identification (gram straining)

Bacterial culture was taken in a Petri dish using an ose needle, then transferred into a glass preparation, then drops of violet crystals was added and kept for 1 minute. After that it was rinsed with distilled water and dried, then drops of iodine was added and kept 1 minute then rinsed with distilled water and dried then dipped in ethanol for ±20 minutes and then one drop of safranin was added and kept for 30 seconds, then rinsed and dried and observe the shape of bacteria under microscope.

Biochemical properties

The gas test was carried out by inserting LAB isolates in 5 mL of BRS BRC MERCK. Then durham tube was inserted in upside down position then incubate for 48 hours at 37°C and checked for the of presence or absence of air bubbles in the durham tube. Furthermore, the catalase test was done by scraping the isolation on the glass preparation and then dropping hydrogen peroxide (H_2O_2) 3%. This was then observed by looking at whether or not gas formed on the bacterial review.

Catalyst test is done by taking lactic acid bacteria isolation by using inoculating loop. The isolation is stroked on object glass, and 3% hydrogen peroxide is (H_2O_2) dripped by using 50 µL pipette. Gas formation was observed on bacteria distribution.[34]

Acid resistance test

Acid tolerance was determined with slight modifications in the methods used by Rashid and Hassanshahian (2014).[35] One mL bacterial culture inoculated on 9 mL Broth MRS media and it was incubated at 37ºC for 24 hours with a pH adjustment of 4 (pH adjusted by addition of HCl 5N) incubated for 90 minutes. Furthermore, the dilution was carried out by the method of spread to the MRS media to be incubated at 37ºC for 48 hours. The number of bacteria that can survive was calculated by the Colony Forming Unit (CFU).

Test for resistance to bile salt

Resistance to bile salt was conducted by researcher in which, one ml of bacterial culture inoculated on 9 ml MRS Broth medium incubated at 37ºC for 5 hours with oxgall settings 0.3%, then diluted to 10-6 then planted with the spread method to MRS media so that it was incubated at 37ºC for 48 hours. The number of bacteria that can survive was calculated using cup count method with the Colony Forming Unit (CFU).[36,37]

Antimicrobial activities

Antimicrobial activity was tested using the disk diffusion method with *Escherichia coli O157, Listeria monocytogenes, Listeria innocua and Staphylococcus aureus* ATCC 25923 bacteria.[38] LAB culture of 1 mL was put into sterile Eppendorf then centrifuged at a speed of 10000 rpm for 5 minutes where the supernatant was used for antimicrobial resistance testing. Nutrient Media was prepared as much as 0.4 grams (the general preparation is 20 grams of Nutrient Agar in 1000 ml of distilled water), after that 0.2% of test bacteria that have been enriched are added and then cooled to harden. As many as isolates as possible was made to be tested and labeled. Then 50µL of LAB supernatant was inserted into the well with a micro pipette. Antibiotics of penicillin, ampicillin and kanamycin were added with the same distance between each. After that, it was incubated for 24 hours at 37ºC aerobically. The antibacterial activity of the LAB supernatant was expressed as the diameter of the clear area formed.

Molecular identification

Lactic acid bacteria isolates were cultured in MRS broth at 37°C for 24 hours. Genomic DNA isolation was carried out using Promega KIT (USA). Amplification of polymerase chain reaction (PCR) of '16S rRNA isolates was done using 16S rRNA gene fragments of ~1.5 KB using universal primers. Initial denaturation at 95°C for 5 minutes with 25 cycles followed by denaturation at 94°C for 1 minute, then annealing at 56°C for 1 minute, extension at 72°C for 1.5 minutes, and final extension at 72°C for 7 minutes. The resulting DNA was separated using electrophoresis at 100 V for 21 min, using 1% agarose in × 1 TAE buffer. Then, a gel documentation system was used to generate an image of the tape. Purification was carried out using a fast gene gel/PCR extraction kit (Promega, USA), and the resulting sequences were analyzed using the BLAST program in the NCBI gene bank database which can be seen at http://blast.ncbi.nlm.nih.gov/Blast.cgi. Sequence alignment was made using the Bioedit application, and phylogenetic trees were created using the MEGA 7 application.

Results

Isolation and identification of lactic acid bacteria

Identification and isolation of *Dadiah* obtained from Halaban.

Table 1. Identification of Lactic Acid Bacteria through microscopic.

Lactic Acid Bacteria Isolation	Color	Shape of Colony	Edge	Elevation
1DA	Yellowish white	Circular	Flat-Smooth	Glistening- Convex
2DA	Yellowish white	Circular	Flat-Smooth	Glistening- Convex
3DA	Yellowish white	Circular	Flat-Smooth	Glistening- Convex
4DA	Yellowish white	Circular	Flat-Smooth	Glistening- Convex
5DA	Yellowish white	Circular	Flat-Smooth	Glistening- Convex

From the result of isolation and purification of lactic acid bacteria a total of lactic acid bacteria contained in *Dadiah* is $44x10^9$ CFU/g. This result is corresponding with criteria by FAO (2002)[38] where the result of colony of lactic acid bacteria that has potential as probiotic is around 10^6 - 10^8 CFU/gram (Table 1).

Characteristics of lactic acid bacteria from Dadiah

The examinations of characteristics of LAB from Dadiah are resistant to gastric acid test, resistance to bile salt test, and antimicrobial activity test. The result of this study is shown Table 2.

Biochemical test

The biochemical test of isolation of *dadiah* by catalyst test and fermentative type yield result that the isolation are negative catalysts and homofermentative type. According to study by another researcher[39] from the catalyst test on *dadiah* culture isolation originated from Lintau, there are no bubbles produced when the isolation is dripped with 3% H_2O_2. It was found that the result of catalyst reaction gave positive result if bubbles are produced which indicate the formation of O_2 gas from the breakdown of H_2O_2 by catalyst enzyme of the bacteria.[40] This is in accordance with study by Harun *et al.*[13] that found LAB isolation produced by *dadiah* from cold water has negative catalysts and Juliyarsi *et al.* (2018)[9] found that LAB isolation produced by *Tempoyak (condiment made from fermented durian)* also negative catalysts.

Resistance to gastric acid

The viability result of resistance to acidic condition (pH_3) test on lactic acid bacteria yield 58.62 – 65.98%. 2DA isolation has the highest viability value of 65.98%. Each isolation has different viability value due to the difference in ability to withstand pH of gastric (acid; Table 3).

Resistance to bile salt

The result of resistance of lactic acid bacteria to 0.3% bile salt for 4 hours yielded viability of 46.67-54.90% with 2DA isolation having the highest viability value among the isolations. This shows LAB isolation can live in bile salt concentration of human body, which is 0.3% (Table 4).

Antimicrobial activity

Selection of probiotic potential in the previous test, obtained 2DA and 4DA isolates with the best results, then screening was done for antimicrobial activity.

The result of this study can be seen in Table 5 which is shown in the area of clear zone or resistance zone for each isolation are varied due to the difference of ability of each bacterium. Based on the microbial activity test, it is found that clear zone of lactic acid bacteria from *dadiah* are formed on *E. Coli* O157, *Listeria innocua*, *Staphylococcus aureus*, and *Listeria monocytogenes* tests. This result shows antimicrobial activity on combatting

Table 2. Biochemical analysis of lactic acid bacteria.

LAB isolation	Catalyst Test	Fermentation Type
1DA	Negative (-)	Homofermentative
2DA	Negative (-)	Homofermentative
3DA	Negative (-)	Homofermentative
4DA	Negative (-)	Homofermentative
5DA	Negative (-)	Homofermentative

Table 3. Viability of resistance to gastric acid.

LAB Isolation	(CFU/mL)		Viability (%)
	pH control	pH 3	
1DA	88×10^7	54×10^7	61.36
2DA	97×10^7	64×10^7	65.98
3DA	114×10^7	72×10^7	63.16
4DA	135×10^7	88×10^7	65.19
5DA	87×10^7	51×10^7	58.62

Table 4. Viability of resistance to bile salt.

LAB Isolation	(CFU/mL)		Viability (%)
	control	pH 3	
1DA	87×10^8	43×10^8	46.67
2DA	102×10^8	56×10^8	54.90
3DA	45×10^8	21×10^8	46.67
4DA	176×10^8	91×10^8	51.70
5DA	$165x\ 10^8$	77×10^8	46.67

Escherichia coli O157 (resistance zone 21.16 mm), *Listeria innocua* (resistance zone of 19.24 mm), *Staphylococcus aureus* (resistance zone of 23.17 mm) on isolate 2DA and *Listeria monocytogenes* (resistance zone of 18.23 mm) on isolate 4DA.

The result obtained from this study (Figure 1) shows that the results of amplification of DNA 1533bp on agarose gel. This indicates that the specific primers used in this study might be able to identify bacteria up to the strain-level.

Based on the result of analysis by using BLAST on https://blast.ncbi.nlm.nih.gov/Blast.cgi, it is found that the type of bacteria of 2DA isolation of fish sauce is kin to *Lactobacillus plantarum* subp. Plantarum Cap Z. Phylogenetic tree obtained shows close kinship among the *Lactobacillus plantarum*. This study differs from research by Amelia *et al.*[38] isolation dadiah from dadiah lintau has result *L. fermentum*, same study conducted by Nurnaafi *et al.* (2015)[41] found that the identification of lactic acid bacteria from Bekasam (traditional foods in South Sumatra) of parrot fish is *Lactobacillus plantarum* and Harun *et al.* (2020)[12] *L. plantarum*

isolation LAB from Cold Water Dadiah.

The variations of LAB in production samples by the two methods observed are influenced by several factors. Deeper study on evaluating the factors causing the variation of LAB from *Dadiah* needs to be conducted in the future. Different methods, recipe, raw material, temperature, and location also might be the cause of the variation in strains.[36] Furthermore, anthropogenic variable (such as the competition of seller and organoleptic preference) is important in producing microbial community structure.[42]

Discussion

Isolation and identification of lactic acid bacteria

From the result of this study shown in Table 1 through microscopic observation (shape, size, and color), it is found that the lactic acid bacteria have yellowish white color with circular shaped colony and glistening-convex elevation. This is in accordance with finding by Purwati *et al.* which the lactic acid bacteria isolation will produce colony with yellowish white color in MRS agar.[43] Each of the isolation is morphological characteristics of lactic acid bacteria according to Suryani *et al.* which stated that LAB isolation will grow when incubated at 37°C, the colony of *Lactobacillus* is characterized by glistening white color, clear zone around the colony, size around 0.5–2 mm, circular in shape and non-fibrous.[44]

Hereafter, the lactic acid bacteria are observed microscopically with gram stain. The gram stain is used to determine whether the bacteria are gram-positive or gram- negative, which will be marked by the absorption of crystal violet and safranin by bacteria. Based on the characteristic of gram stain (microscopic), it is found that 1DA, 2DA, 3DA, 4DA, and 5DA are bacilli (rod-shaped) and gram-positive (purple colored). This result is in accordance with Salminen *et al.,* who found gram-positive, rod-shaped or circular-shaped, facultative anaerobe bacteria that do not produce spores, and producing lactic acid as main product of carbohydrate (glucose, fructose, and sucrose) fermentation are characteristics of lactic acid bacteria.[45] Unus stated that gram-positive bacteria which are given crystal violet will stay purple colored after being washed in alcohol and stained by red safranin, whereas gram- negative bacteria will change color into red.[46] The result of previous study reported the morphology characteristics of lactic acid bacteria produced from milk fermentation. Sunaryanto and Marwoto stated that lactic acid bacteria isolation from *dadiah* are gram-positive, bacilli, and do not produce spores.[47] Purwati *et al.* found that 11 lactic acid bacteria isolation from *dadiah* originated from Air Dingin District, Solok, are bacilli and gram-positive bacteria.[48] Nur *et al.* found that lactic acid bacteria isolation produced from *Dangke (cheese)* are bacilli and gram-positive.[49] Amelia *et al.* found *dadiah* originating from Lintau also produced lactic acid bacteria isolation which are gram-negative and bacilli.[39]

Characteristics of lactic acid bacteria from dadiah

The fermentation type is determined as homofermentative

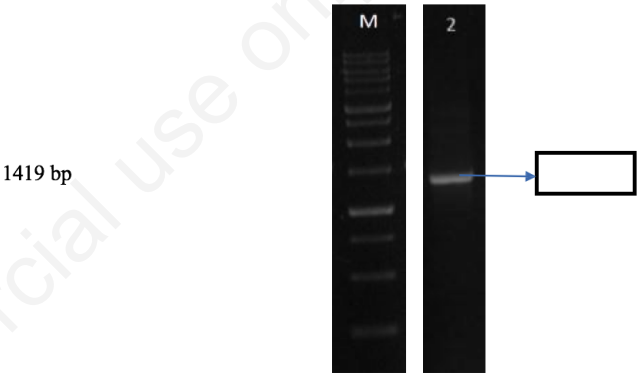

Figure 1. PCR amplification of 2DA sample

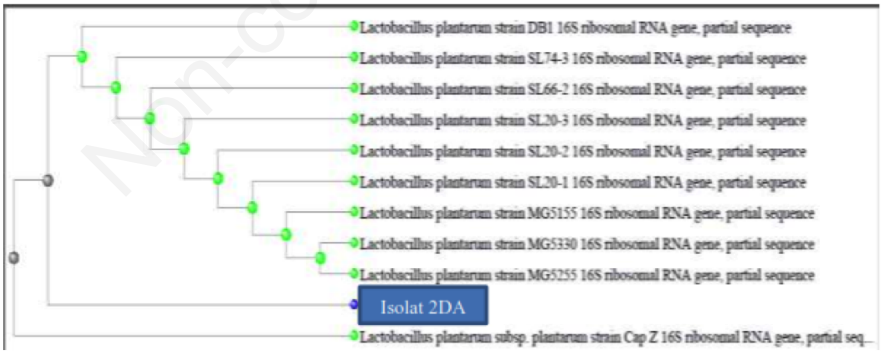

Figure 2. Phylogenetic analysis of 2DA isolation.

Table 5. Antimicrobial activity of LAB from dadiah.

Source of Resistance	Clear Zone (mm)			
	E. coli O157	*Listeria innocua*	*Staphylococcus aureus*	*Listeria monocytogenes*
2DA	21.16	19.24	23.17	11.16
4DA	17.24	15.2	15.2	18.23
Ampicillin	-	-	5.05	-
Kanamycin	14.95	16.80	15.20	14.10

when there are no bubbles produced inside durham tube. It was found that isolation from *dadiah* originated from Lintau also included as homofermentative type, where the homofermentative type will produce lactic acid as primary metabolism product.[39] In addition, the test of biochemical characteristic on strain *Pediococcus acidilactici* PB22 lactic acid bacteria isolation produced by *bekasam* are also homofermentative type.[11]

Resistance to gastric acid

As a probiotic candidate, lactic acid bacteria must be able to withstand gastric acid with pH 2–3. Jain *et al.* found that isolation from sample of cow milk and feces can withstand pH 3 (61.44–81.25%).[50] Another study found that strain *Lactobacillus fermentum* L23 lactic acid bacteria isolation can withstand pH 2 with viability of 55.64–73.94%.[51]

The result on 2DA isolation from *dadiah* is higher than *Lactobacillus rhamnosus*

which is isolated from buffalo milk originated from Karnataka, India, which has viability of 30% under pH 3 for 3 hours.[52] Some researcher stated that the higher the viability of lactic acid yielded, the higher the resistance of the bacteria or lactic acid bacteria isolation to gastric acid.[11]

Resistance to bile salt

The isolations will show difference on viability to 0.3% bile salt (Table 4) after 5 hours of incubation. 2DA isolation (54.90%) shows the highest resistance compared to other isolations. The level of viability of resistance of bacteria to bile salt will influence the potential of LAB as probiotic. Several probiotic bacteria have been proven able to live in this condition. The result of 2DA isolation is higher than strain *Lactobacillus fermentum* IMAU70167 isolation from buffalo milk that can withstand 0.3% bile salt for 4 hours with viability of 32.23–56.13%.[50] Another study by Anandharaj and Sivasankari found stain *Lactobacillus oris* HM168 isolated from breast milk can withstand oxgall 0.3% for 3 hours with viability 20.1–26.9%.[53] According to another researcher, the resistance of lactic acid bacteria to bile salt is related to Bile Salt Hydrolase (BSH) enzyme which helps to hydrolyze conjugated bile salt in order to lessen the effect of toxin on cells.[11]

Antimicrobial activity

This result is higher than 2DA isolation which shows it is the most effective on *E. coli* O157, *L. innocua and S. aureus* bacteria test. This study on antimicrobial and lactic acid bacteria of 2DA isolation on *E. coli* O157 test is higher than the study conducted by Juliyarsi *et al.* with has resistance zone of lactic acid bacteria isolation from *tempoyak* on O157 testing bacteria, which is 12 mm.[9] Whereas the result of antimicrobial and lactic acid bacteria on *Listeria monocytogenes* testing bacteria in this study has relatively lower resistance than the study by Melia *et al.*[11] The indigenous microbes, including LAB and yeasts, found in buffalo milk and bamboo tubes also helps in the formation of flavor and desired texture of dadih. More studies are required in this field to find the potential health benefits and improve the sensory quality of dadih.[54]

Conclusions

The isolation of lactic acid bacteria from *dadiah* of Halaban with total of $44x10^9$ CFU/gr colonies from the five isolations isolated are known to be gram-positive bacteria with negative catalyst, homofermentative type. Resistance to gastric acid with pH 3 with viability of 65.98%, resistance to 0.3% bile salt with viability of 54.90% from 2DA isolate. The best result of clear zone area from antimicrobial activity is obtained from 2DA isolation with testing bacteria including *Eschericia coli* O157 with area of 21.16 mm, *Staphylococcus aureus* with area of 23.17 mm, *Listeria innocua* with area of 19.24 mm, and *Listeria monocytogenes* with area of 18.23 mm on 4DA isolate. Identification of LAB is by using 16S Srna gene, the result of running PCR is base length of 1419 bp. From phylogenetic analysis, it is found that LAB from *dadiah* of Limapuluh Kota Regency is kin to *Lactobacillus plantarum*. The superiority of identification technology of LAB based on 16S rRNA gene only can be conducted if the nucleotide sequence information of the targeted bacteria is known beforehand. Phylogenetic analysis shows that dadiah of Limapuluh Kota Regency is kin to *Lactobacillus plantarum*. The superiority of identification technology by using 16S rRNA gene only can be conducted if the nucleotide sequence information of the targeted bacteria is known beforehand.

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