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Molecular Analysis of Lactic Acid Bacteria Isolated from *Pado* Fish of Agam Regency, West Sumatra Indonesia as Probiotics

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ABSTRACT

Pado is a typical fermented fish from West Sumatra with a mixture of *Simauang* seed meat (*Pangium edule* Reinw) and coconut dregs covered with plastic for 4 to 8 days. *Pado* has good nutritional value and is thought to have a lactic acid bacterial activity to be used as a probiotic-producing functional food. Therefore, this study aims to determine the molecular isolation and identification of LAB types found in *Pado* fish using the 16S rRNA method. The research method is a descriptive method and laboratory analysis. The sample used as material for this research is *Pado* fish from Lubuk Basung District, Agam Regency, West Sumatera. The analysis related to probiotic characteristics includes total colonies of lactic acid bacteria, gram staining, acid resistance, resistance to bile salts, antimicrobial activity using test bacteria *Escherichia coli* O157, *Staphylococcus aureus*, *Salmonella* sp, and *Listeria monocytogenes*, as well as molecular sequential analysis using the 16S rRNA gene and phylogenetic diversity analysis using MEGA software version 7.0. The results showed that the total lactic acid bacteria is $7.3x10^7$ CFU/gr; the seven isolates will be identified as gram-positive. Viability to gastric pH resistance ranged from 7.63-61.31%, and viability to bile salt resistance ranged from 22.86-71.05%. Three isolates had antimicrobial activity against *E. coli*, *S. aureus, Salmonella* sp., and *L. monocytogenes*. Molecular analysis using the 16S rRNA gene showed that PDY1 isolate is similar to *Pediococcus acidilactici*.

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1- Introduction

Indonesia is an archipelagic country, and almost one-third of the country's area consists of the sea, so there are many processed foods based on seafood and fisheries. Fermented food processing is a traditional process that we still find today. West Sumatra is one of the regions with culinary diversity that has a distinctive flavor and is healthy for the body. Especially foods that go through the fermentation process, such as Budu fish, Pado cincalok, boyom, and tukai from West Sumatra. Pado is a local resource originating from West Sumatra made from fresh fish that is fermented using a mixture of simauang seeds (Pangium edule Reinw) and coconut pulp stored in an aerobic state for 4-8 days.[١]

The fresh fish used for this product is marine fish, which includes mackerel (Rostrelliger faughni), Lemuru or Spotted Sardinella (Ambligaster sirm), Tenggiri (Rastrelliger brachysoma), and Kwe or Yellowtail Scad (Atule sp. A) [2]. Pado can currently be found in Agam Regency, West Sumatra, around the Lubuk Basung and Maninjau areas. The product is widely sold in traditional markets in Bukittinggi and Lubuk Basung cities and around Maninjau and Sungai Limau districts.[٣]

The fermentation process of these local foods will produce Lactic Acid Bacteria (LAB), which can potentially be probiotics. Probiotics, if consumed in sufficient quantities, can maintain the health of the body [4]. Probiotic bacteria that enter the digestive tract will attach to the small intestine, balancing the microflora and facilitating the absorption of nutrients.[٥]

Also, fermented foods are made from fish, such as scabs found in the South Sumatra, Java, and South Kalimantan areas. Some LAB isolation results from fermented fish such as Pediococcus acidilactici from bekasam [6], Lactobacillus brevis from budu fish [7], Lactobacillus casei from bekasam [8], Lactobacillus plantarum from pado fish.[١]

Pado has good nutritional value and is thought to have a lactic acid bacterial activity to be used as a probiotic-producing functional food. According to the International Scientific Association for Probiotics and Prebiotics [9], probiotics are living organisms that benefit their hosts' health if consumed in sufficient quantities. The criteria for probiotic microorganisms that have been established by the FAO/WHO [10], include being able to survive in conditions of gastric acid and digestive bile salts, providing benefits to the intestines, being able to stick to intestinal mucus or epithelial cells, producing antimicrobial activity against pathogenic bacteria, are Microorganisms that are safe or including GRAS microorganisms (generally recognized as safe), do not produce toxins, are not resistant to antibiotics [11,12], and are not pathogenic bacteria. Researchers aim to find out the LAB of pado fish from the Agam area, West Sumatra, as a probiotic, especially those with potent antimicrobial activity molecularly.

2- MATERIALS AND METHODS

1 -Materials

This study used materials, namely Pado, from three locations in Lubuk Basung District, Agam Regency, West Sumatra, Indonesia .

2 -Methods

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Pado fish were brought in an ice box in sterile conditions, then taken and analyzed at the Animal Products Technology Laboratory, Faculty of Animal Science, Andalas University.

This research was carried out experimentally with descriptive interpretation of the results by looking at the average of each replication carried out.

2 -٣ -Parameters

3 -1- Isolation and purification of LAB

The bacterial culture that has been enriched is taken as much as 100 µl and transferred to Eppendorf containing 900 µl of MRS Broth. Do dilutions until 10-7. The 10-7 dilution results were taken 100 µl of the sample and planted on a Petri dish that already contained MRS media so that it was then leveled with a hockey stick. The inoculum was stored in an anaerobic jar and incubated for 48 hours at 37°C. After 48 hours, a single colony that characterized LAB, namely round yellowish-white slippery, was transferred to MRS Agar media for colony purification using the streak method, using an ose needle, and then incubated for 24 hours at 37° C.[١٣]

3 -2 -Gram Staining

A bacterial culture is spread on a glass object (preparation) cleaned with alcohol. Dry it on a bunsen or dryer. It dripped with crystal violet dye. Then, wait for 1 minute for the bacteria to absorb the stain. Then rinse under running water, dabbed with iodine complex solution, wait for 1 minute, and rinse with running water. Wash with alcohol by dipping in diluted alcohol. Dripped with safranin dye, then waited 30 seconds. After that, it was dried and examined under a microscope [14] (Dwidjoseputro, 1989).

3 -3 -Acid Resistance Test

A 500 µl LAB culture enriched for 24 hours is put into a test tube containing 5 ml of MRS Broth. Drop HCl into the solution until it shows a pH of 3, which is the stomach acid's pH—then incubate for 90 minutes. Dilute the solution to 10-6 dilution. The result of 10-6 dilution is planted 100 µl into a petri dish containing MRS agar media—Incubation for 48 hours, and calculation of the colony and LAB viability.[١٥]

3 -4 -Resistance Test to Bile Salt

One ml of bacterial culture was inoculated on 9 ml MRS Broth media and incubated at 37 ºC for 24 hours. Furthermore, it took 1 ml of bacterial culture into a reaction tube containing 9 ml of MRS Broth without ox gall setting (control), and on MRS Broth, 0.3% incubated ox gall setting was for 5 hours. Then dilution was carried out to 10-6 and planted with the spread method onto MRS Agar media, incubated at 37ºC for 48 hours. The number of bacteria that can survive is calculated using the plate count method with the Colony Forming Unit (CFU), and the LAB viability is calculated.[١٥]

3 -5 -Antimicrobial Activity

One ml of LAB culture previously enriched was taken using a micropipette and put into Eppendorf. Then centrifuged at 10,000 rpm for 5 minutes, using the supernatant for the antimicrobial resistance test. Nutrient media To prepare 0.4 grams after the medium has cooled slightly (± 45ºC), it is poured into a petri dish of ± 20 ml, added with 0.2% of the test bacteria (Escherichia coli O157, Staphylococcus aureus, Salmonella sp, and Listeria monocytogenes) have been enriched for 24 hours, and homogenized. After the order hardens, many isolates will be made to be tested. Then, 50 µl of LAB supernatant into the well. After that, it was incubated for 24 hours at 37ºC aerobically. After 24 hours, observations were made of the clear zone

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formed by measuring the diameter using a caliper.[١٦]

3 -5 -Molecular Analysis Sequencing

Lactic acid bacteria isolates were cultured in MRS broth at 37°C for 24 hours. Genomic DNA isolation was carried out using the Promega Kit (USA). 1000 µL of colony lactic acid bacteria isolates from MRS Broth were taken and put into a new Eppendorf, centrifuged at 14000 rpm for 2 minutes. Then, the supernatant is removed, and the pellets are taken—then 120 µL of Lysozyme—next, Incubation in a 37ºC water bath for 60 minutes. Centrifuge for 2 minutes at 14000 rpm, then the supernatant is removed, and the pellets are taken and added with a 600 µL nuclei lysis solution. Incubated at 80ºC for 5 minutes, then let stand at room temperature. 3µL of RNase solution was incubated in a 37ºC water bath for 60 minutes. The 200 µL of protein precipitation solution is added and then vortexed. Add 600 µL of isopropanol.

Furthermore, centrifuged for 2 minutes at 14000 rpm, the pellets are taken, and the supernatant is removed. Add 600 µL of 70% ethanol, then homogenized. The pellet is centrifuged for 2 minutes at 14000 rpm, the pellets are removed, and the supernatant is removed. Rehydrate DNA pellets by adding 10-100 µL of Rehydration solution for 30 minutes at 65ºC.

Primer R (16S-1492R, Tm 47°C, 5'-GTT TAC CTT GTT ACT ACT-3 ') and F (16S-27F, Tm 54.3°C, 5'-AGA GTT TGA TCC TGG CTC AG-3'), prepared (10 µm concentration). Take 90µL dH2O + 10µL (Primary R and F). (R and F primers in TE buffer (100 µM concentration) Eppendorf in1 PCR Cocktail (12.5 µL Master Mix, one µL F Primer, one µL R Primer, one µL DNA Template, 9.5 µL ddH2O), denatured PCR 95 ° C 45 seconds, 56 ° C annealing 45 seconds, 72°C extension 1 minute 40 seconds, 72°C final extension 10 minutes Electrophoresis 10 µL sample into agar well, insert four µL DNA ladder. Set to 100 V for 45 minutes. The gel was placed in a container added with TBE until submerged. A UV lamp can view the gel. The 16S rRNA gene sequences from the isolates were submitted to NCBI for a BLAST search. The phylogenetic tree was manufactured by MEGA version 8.0.

RESULTS AND DISCUSSION

1 -Total Lactic Acid Bacteria

The sample used is pado fish taken from the Lubuk Basung area, Agam Regency. It obtained a total LAB of 7.3x107 CFU/gr, indicating that this pado fish fermentation product is included as a functional food as a source of probiotics. This is in accordance with the provisions of FAO/WHO [10], which state that functional foods as a source of probiotics must have a minimum total number of LAB colonies of 107 CFU/ml/gr.

Then, microscopic observations were made by looking at the cell shape of each colony; it was found that all colonies were gram-positive, three were coccus-shaped, and four were basil-shaped; observations were made using a microscope with 100x magnification. This is by the opinion of Irianto [17], that lactic acid bacteria are gram-positive bacteria. Salminen et al. [18] that LAB is a facultative anaerobic bacteria, gram-positive, rod-shaped, or round, does not produce spores and produces lactic acid, which is the main product of carbohydrate fermentation (glucose, fructose, and sucrose).

2 -Resistance Test of Lactic Acid Bacteria against Acids

For further analysis, seven LAB colonies were taken to test the characteristics of probiotics by looking at

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the resistance of bacterial colonies to gastric pH conditions.

Table 1. LAB resistance to acid condition

LAB Isolate Samples	Number of Bacterial Cells (10^7 CFU/ml)		Viability of LAB (%)
	pH control	pH 3	
PDY1	168	103	61.31
PDY2	79	12	15.19
PDY3	167	32	19.16
PDY4	56	32	57.14
PDY5	118	9	7.63
PDY6	167	45	26.95
PDY7	95	54	56.84

The results showed that each isolate had a different viability. This was because each isolate had different abilities to survive at low pH. This occurs because the differences in cytoplasmic membranes are other. The characteristics and membrane permeability influence this diversity. Cotter and Hill [19] stated that LAB has different acid tolerance due to species' relative permeability differences.

Resistance to stomach acid is also an essential requirement for an isolate that can become a probiotic. This is because if the isolate enters the human digestive tract, it must withstand stomach acid [20]. It can categorize LAB isolates from Pado fish as probiotics because they can survive at acid pH. LAb resistance ability as one of the selections that bacteria must go through until they reach the intestine must be able to survive up to a minimum number of colonies of 106 Log CFU.[٢١]

3 -Resistance Test of Lactic Acid Bacteria to Bile Salt

Table 2. LAB resistance to bile salts

LAB Isolate Samples	Number of Bacterial Cells (CFU/ml)		Viability of LAB (%)
	Control	*Ox gall* 0,3% (Ox)	
PDY1	112	48	42.86
PDY2	35	8	22.86
PDY3	119	76	63.87
PDY4	76	54	71.05
PDY5	86	35	40.70
PDY6	121	67	55.37
PDY7	65	25	38.45

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The results of the LAB resistance to bile salts showed that PDY4 had a high viability of 71.05%. This indicated that all isolates from pado were resistant to bile salt conditions. Amelia et al. [22] states that some LABs have the enzyme bile salt hydrolase (BSH), which is active in hydrolyzing bile salts. This enzyme can change bile salts' physical and chemical abilities so that they are not toxic to LAB. This is what may be the cause of LAB resistance to the state of bile salts .

4 -Antimicrobial Activity

The results of further testing selected three candidates with the best pH and bile salt resistance to see the antimicrobial activity of each LAB isolate.

Table 3. Measurement of clear zone diameter for antimicrobial activity of LAB isolates

Inhibitory Source	The Size of Clear Zone (mm)			
	E. coli 0157	*Staphylococcus aureus*	*Salmonella sp.*	*Listeria monocytogenes*
PDY1	14.65	16.67	17.43	9.96
PDY4	9.76	12.43	18.75	11.65
PDY7	10.54	16.98	16.21	4.87
Ampicillin	22.27	-	-	-
Kanamycin	17.22	14.7	12.2	6.11
Penicillin	-	-	-	-

The research showed that LAB from Pado formed a clear zone against E. coli O157, Staphylococcus aureus, Salmonella sp, and Listeria monocytogenes test bacteria. Melia et al. [23] stated that lactic acid bacteria from honey could inhibit Listeria monocytogenes and E. coli. The results are better when compared with pado isolates from the Balingka area, Agam Regency, where the inhibition of E. coli and L. monocytogenes bacteria is 5.10 and 9.14 mm.[١]

Pelczar and Chan [24], added that the larger the clear zone formed, the greater the inhibitory activity of lactic acid bacterial isolates against pathogenic bacteria. In penicillin antibiotics, no clear zone is formed. It can be caused by enzymes that can damage the drug's working power, changes in the locus in bacterial cells that target the drug, and changes in the bacterial metabolic pathway.[٢٥]

5 -Analysis of 16S rRNA Gene Sequence Isolate from Pado

We can see an image of the PCR sequencing of Pado PDY1 isolates in the Figure below.

[DOI: 10.22034/FSCT.21.155.1]

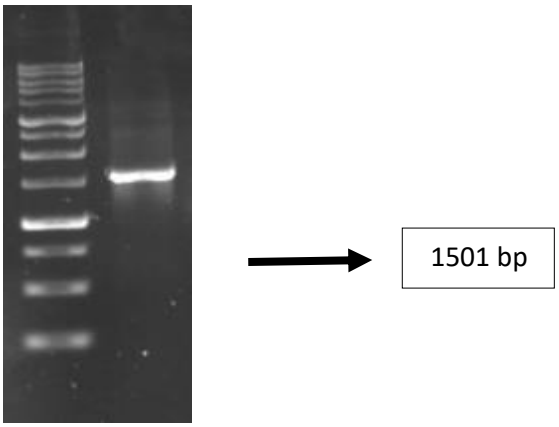

Figure 1. PCR Results of BAL *Pado* (PDY1) Isolation Sequence

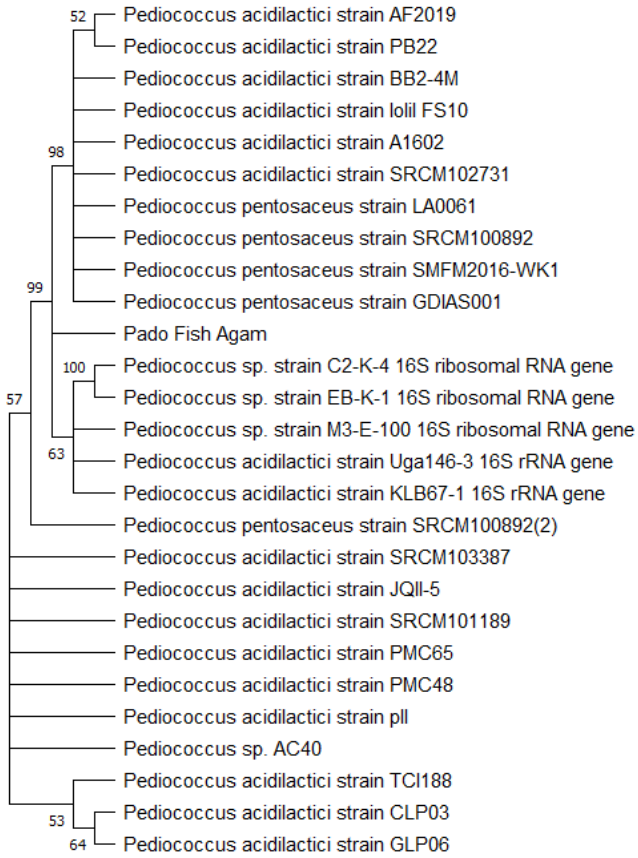

Figure 2. Phylogenetic Tree of BAL *Pado* Isolat

Sequencing and BLAST analysis showed that the LAB isolates bacteria from Pado were similar to the Pediococcus acidilactici strain AF2019. The phylogenetic tree shows that the closest distance to Pado isolates is Pediococcus acidilactici. Hagstrom et al. [26], stated that in isolates with similar 16S rRNA sequences, more than 97% could represent the same species. In contrast, the sequence similarities between 93-97% represent bacteria's identity at the genus level but different species. The results of this study are very different from those conducted by

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Syafitri et al. [27], using molecular analysis on pado fish with the 16S rRNA gene found that some of the isolates obtained were Lactobacillus pentosus and Lactobacillus plantarum. Likewise, Annisah et al. [1] found that identifying pado fish obtained Lactobacillus plantarum.

CONCLUSION

LAB Pado isolates had total LAB colonies ranging from. LAB isolate from Pado is resistant to gastric pH and bile salts. Lactic acid bacteria isolates in pado can inhibit pathogenic bacteria's growth, including Escherichia coli O157, Staphylococcus aureus, salmonella sp., and Listeria monocytogenes. The sequencing results of the LAB Pado PDY1 isolate from the Labuk Basung District, Agam Regency, were Pediococcus acidilactici.

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