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Characterization, molecular identification and antimicrobial activity of lactic acid bacteria with potentials as halal probiotics isolated from Rinuak fish (*Psilopsis sp.*) in Lake Maninjau, West Sumatra, Indonesia

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Abstract

Background and Objective: The exploration of lactic acid bacteria in integration of specific halal certification is one of the major research topics in the fields of health, food, animal husbandry and agriculture. This study aimed to investigate antimicrobial potentials of probiotic lactic acid bacteria isolated from Rinuak fish (*Psilopsis* sp.) from Lake Maninjau, Indonesia.

Material and Methods: Totally, 15 lactic acid bacteria were isolated from four samples of Rinuak fish (*Psilopsis* sp.) and investigated for their characteristics as probiotic candidates using conventional laboratory assessments and 16S rRNA sequencing methods.

Results and Conclusion: Five isolates were identified as probiotic candidates, including IR2.2, IR2.4, IR4.1, IR4.3 and IR4.5 due to their good resistance of gastric pH ranging 84.24–88.01% and their survival ability against bile salts (resistance of 50.37–57.35%). The IR4.3 was identified to generate the greatest antimicrobial activity against *Escherichia coli* ATCC 0157, *Staphylococcus aureus* ATCC 25923 and *Salmonella* enteridis ATCC 13076 with their diameter of inhibition zone of 22.46, 19.34 and 9.41 mm, respectively. The 16S RRNA sequencing method verified that the lactic acid bacteria isolated from rinuak fish (*Psilopsis* sp.) included 97.69% similarity to *Lactobacillus fermentum* strain 4901. This strain promised as an antidiarrheal and antityphoid agent and a natural food preservative appropriate for incorporation into HALAL-compliant foods and pharmaceutical products.

Keywords: Food preservative, Endemic Fish, Microbial fermentation, Molecular characterization, *Lactobacillus fermentum* strain 4901، Indigenous probiotics،Food microbiology،Gastrointestinal health

What is "already known" on this topic:

- Lactic acid bacteria (LAB) are widely known to have probiotic potential and can be isolated from fermented foods, including fish.
- The Rinuak fish (Psilopsis sp) from Lake Maninjau contains high protein and mineral content (e.g., calcium, magnesium) that may support LAB growth.
- Prior studies had not comprehensively explored the antimicrobial properties or probiotic potential of LAB specifically isolated from fermented Rinuak fish.

What this article adds:	➢ Identification of Lactobacillus fermentum strain 4901 as a halal probiotic candidate from Rinuak fish. ➢ Superior antimicrobial activity surpassing standard antibiotics against key pathogens. ➢ Potential applications in halal food preservation and therapeutic products. ➢ First comprehensive study on probiotic LAB from an endemic fish species in Lake Maninjau.

1. Introduction

Global challenges in various aspects of life are becoming larger. This can be seen from the increasing human population and hence demands for food products are increasing as well; one of which, is fishery products. This fishery product is a basic requirement to meet the needs of protein sources. The protein must be ensured that is halal due to the tauths of the Islamic religion, because Islam emphasizes that maintaining a healthy body by consuming halal foods and drinks is an obligation for every muslim [1]. For halal food development, it is essential to ensure that all ingredients and processes comply with Islamic dietary laws. Lactic acid bacteria (LAB) sourced from permissible animals such as fish and processed without contamination from non-halal substances can be addressed as halal. However, clear certification is warranted for commercial uses. Fermented fish is one of the products from fisheries, which is rich in proteins and LAB [2]. In the fermentation process, microbes and enzymes can stimulate specific flavors, increase the digestibility of food ingredients, decrease the content of anti-nutrients and other undesirable ingredients and produce derivative products and compounds that are beneficial for human life [3]. In general, LAB are food-grade microorganisms. These bacteria can provide flavors to foods, inhibit spoilage bacteria in foods and inhibit pathogenic bacteria. The LAB can be isolated from various sources, especially from fermented foods [4]. In addition, LAB can create an acidic atmosphere, which can decrease the number of pathogen colonies [5]. The existence of selected strains of LAB has demonstrated beneficial effects, as probiotics for humans [6].

The LAB isolation is possible from plant and animal-based products; for example fish, fruits and milk [7]. Rinuak fish (*Psilopsis* sp.) of Lake Maninjau, Indonesia, is one of those products. Rinuak fish is an domestic fish of Lake Maninjau that includes animal proteins, which is potentially developed due to its high nutritional compounds. The flesh of rinuak fish (*Psilopsis* sp.) contains proteins of 14.52%, magnesium of 0.21% (in fresh rinuak), phosphorus of 2.4% (in fresh rinuak), water content of 78.62% and ash content of 6.4% (in fresh rinuak). Rinuak fish also contains calcium [8]. The mineral composition especially magnesium and calcium are able to stimulate the bacterial growth. Magnesium (especially in gluconate form) improves probiotic survival, texture, acidity and viability (> 10^6 CFU g^{-1}) during storage [9]. Research by [10] showed that addition of calcium (calcium carbonate) to fermented feed helped boost growth of *Lactiplantibacillus plantarum*, *Lacticaseibacillus rhamnosus* and *Bacillus subtilis*. Therefore, these mineral nutrients of rinuak fish support the potential of rinuak fish for LAB growth. However, there is a lack of studies that investigate its antimicrobial activity and potential characteristics as probiotics.

One of the most important characteristics of LAB is that they can produce the antimicrobial compounds of bacteriocins [11]. Bacteriocins are secondary metabolite products of LAB that include similar actions to antibiotics, being able to inhibit certain bacteria from growing [12]. Previous studies were carried out worldwide to investigate the LAB content in fish. For example, studies on swamp fish fermented with the addition of pure coconut oil resulted in the discovery of LAB with antimicrobial activity [13]. Potential antimicrobials can be achieved from LAB produced by fermentation of rinuak fish (*Psilopsis* sp.) in Lake Maninjau by isolating and characterizing DNA from LAB through polymerase chain reaction (PCR) analysis of 16s rRNA gene and genome sequencing. Based on the results of this molecular analysis, the phylogenetics of LAB from the fermented rinuak fish (*Psilopsis* sp.) of Lake Maninjau was investigated. Numerous previous studies were carried out on the nutritient of rinuak and LAB contents of rinuaks. However, study on the antimicrobial potential of fermented rinuak fish and its probiotic characteristics has not been investigated. Therefore, this study aimed to characterize the isolated LAB morphologically and biochemically and carry out theit molecular identification using 16s RNA sequence method. The probiotics effectiveness in this study was assessed on a strain spesific basis.

2. Materials and Methods

a. Research Equipment and Materials

Equipment of this study included incubator (Infors HT Ecotorn, USA), anaerobic jars, refrigerator, autoclave (ALP KT40S, Japan), hot plate (Sybron nouveau II, USA), vortex (Labmart 3000, China), analytical balance (Kern ABT 320-4M, Germany), bunsen, microtubes, hockey sticks, inoculation needles, glass slides, pipettes and micropippette tips (DragonLab, BIO-RAD, USA), light microscope (Irmeco, USA), UV-vis spectrophotometer, oven (Memmert UF 100 Plus, Germany), centrifuge (Eppendorf 5417 R, Germany), thin layer chromatography (TLC) chamber and paper chromatograph, capilary tubes, 96-well microplates, microplate reader (PR 4100, BIO-RAD, USA), pH meter, shaker waterbath (IC DK-540b, Japan), PCR equipment (Techne TC-312, UK), spinner down (BIO-RAD, USA) electrophoresis equipment (BIO-RAD, USA), gel documantation imager (BIO-RAD, USA) and labobartory standard glasswares.

Materials of this study included rinuak fish sampels collected from Lake Maninjaus, Indonesia, de Mann-Rogosa-Sharpe (MRS) broth and agar (Merck, Germany), distilled water (DW), 70 and 90% ethanol, peptone water, MRS agar (Merck, Germany), 37% HCl (Merck, Germany), ox-gall, nutrient agar (NA), penicillin, kanamycin, ampicillin, NaOH (Merck, Germany), genomic DNA mini kit (Invitrogen Pure-Link, USA) and lysozyme (Invitrogen PureLink, USA)

b. Fermentation of Rinuak Fish (*Psilopsis* sp.) from Lake Maninjau

The process of making rinuak fish fermentation (*Psilopsis* sp.) was as follows. The ingredients were fish, salt and rice. First, fish was washed and then salt and rice were add to nourish the taste. Samples were transferred into a jar and the lid was close tightly. Jars were stored at room temperature (RT) for 4 d and then dried for 3–5 d [14]. The flowchart of the fermentation process for rinuak fish (*Psilopsis* sp.) from Lake Maninjau can be seen in Figure 1.

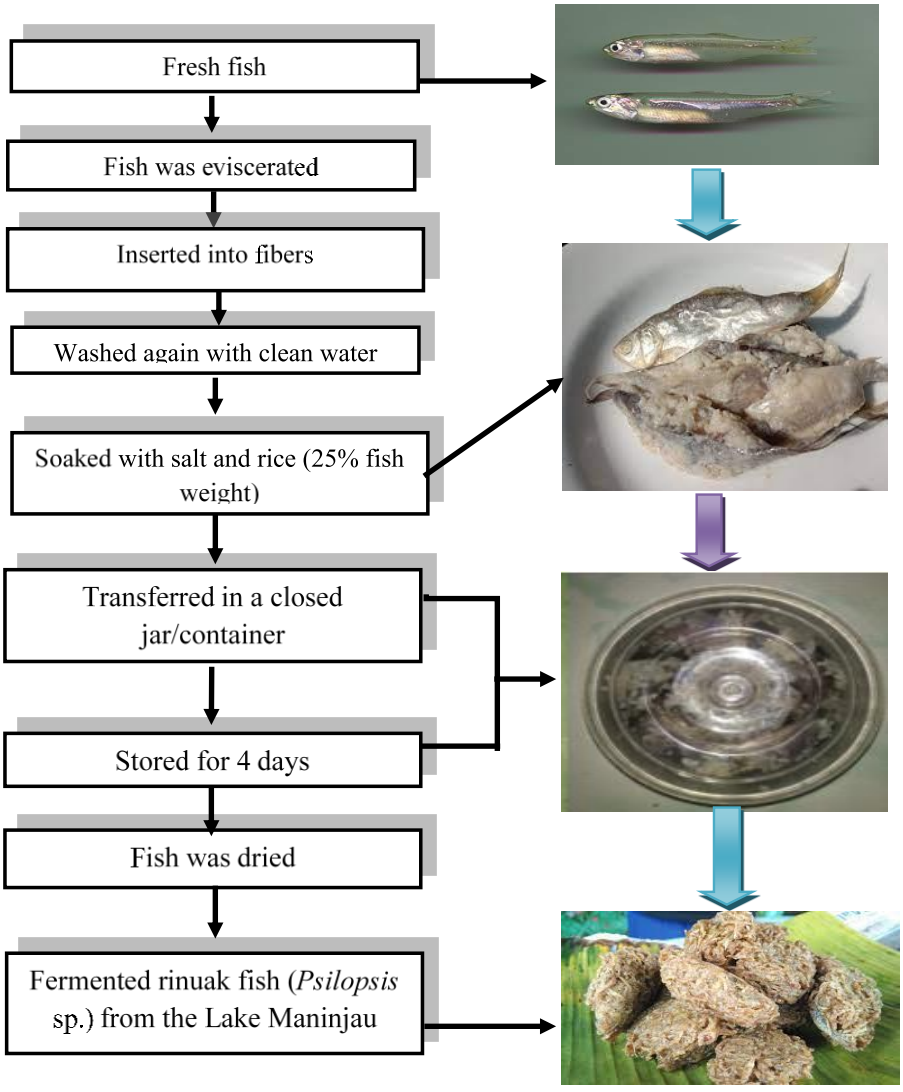

Figure 1. Fermentation process of rinuak fish (*Psilopsis* sp.) by the local community, Lake Maninjau, West Sumatra, Indonesia

c. Sampling Locations for Rinuak Fish (Psilopsis sp.)

Samples of rinuak Fish (*Psilopsis* sp.) were collected from fishermen in Lake Maninjau at various locations based on the easy access to collection locations, residential areas in the lake area, upstream rivers around the lake, depth of the lake and food sources for rinuak Fish. Sample 1 was collected in a dense settlement and included a traditional market in the Lake Maninjau area. Sample 2 was collected from the middle of the lake with a depth of nearly ±150 M. Sample 3 was collected in a tourist attraction area at Lake Maninjau and Sample 4 was collected in the upstream area of the river, the Batang Sri Antokan River; where, there was the Maninjau Hydroelectric Power Plant. Fish was immediately transferred into a cold box and carried to the Microbiology Laboratory of Stikes Syedza Saintika. Then, isolation and molecular characterization of DNA of LAB were carried out at the Biotechnology laboratory of Andalas University, Indonesia. The location of each sample is illustrated in Table 1 and a map showing the locations for rinuak fish in Lake Maninjau, West Sumatra, Indonesia, is demonstrated in Figure 2.

d. Isolation of Lactic Acid Bacteria from Rinuak Fish (Psilopsis sp) Lake Maninjau, West Sumatra, Indonesia

The LAB isolation process began with an enrichment process; 1 g of rinuak fish sample was added into 9 ml of MRS broth and then homogenized to achieve a 10^{-1} dilution. This was incubated at 37 ºC for 24 h. After incubation, 100 µl of the enrichment or 10^{-1} dilution were added into a microtube with 900 µl of peptone water and then homogenized using vortex to achieve a 10^{-2} dilution. This procedure was repeated until 10^{-8} dilutions were achieved. Then, plating process was carried out and the culture from the 10^{-8} dilution was inoculated on MRS agar and incubated anaerobically at 37 ºC for 48 h. Single LAB colonies that grew on the surface of the media were re-purified by inoculating the colony onto MRS agar, incubating anaerobically at 37 ºC for 24 h [15].

e. Characterization of Lactic Acid Bacteria from Rinuak Fish (Psilopsis sp.) in Lake Maninjau, West Sumatra, Indonesia

Identification of lactic acid bacteria morphology

Morphological identification was carried out macroscopically on LAB cultures inoculated on MRS agar to identify the shape, color and diameter of LAB isolates. Then, Gram staining was carried out to investigate LAB morphology microscopically by verifying the color and the shape of the cells. Meanwhile, biochemical characterization was carried out using fermentation-type and catalase assays [15].

Sample Code	Location	Type of Fish	Storage Time (days)
IR 1	Maninjau Village	Rinuak Fish (*Psilopsis* sp.)	2
IR 2	Tanjung Sani Village		4
IR 3	Desa Duo Koto Village		2
IR 4	Desa Koto Malintang Village		3

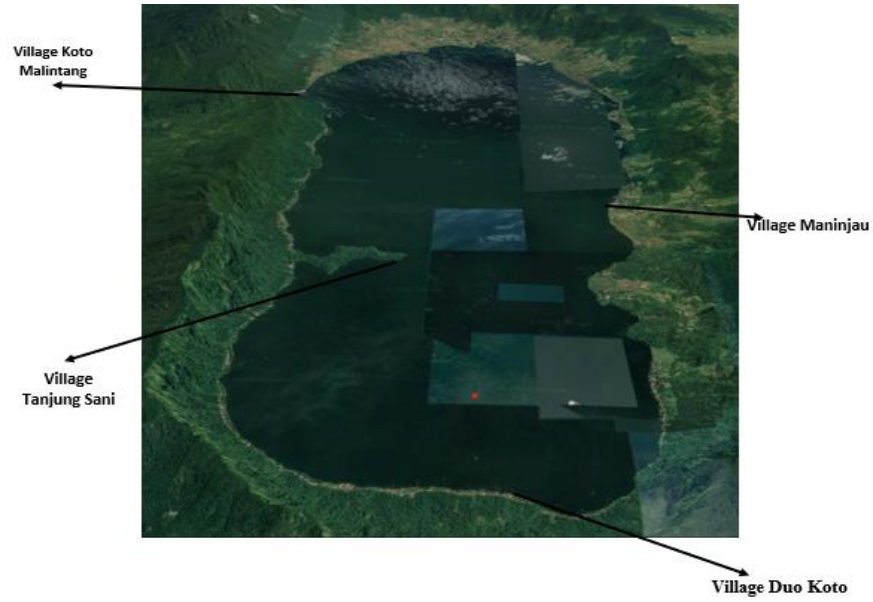

Figure 2. Map of the rinuak fish *(Psilopsis* sp.) collection locations in Lake Maninjau, West Sumatra, Indonesia

f. Selection of Lactic Acid Bacteria from Rinuak Fish (Psilopsis sp) from Lake Maninjau, West Sumatra, Indonesia, as Probiotic Candidates

1) Resistance of Rinuak Fish (Psilopsis sp.) from Lake Maninjau, West Sumatra, Indonesia, to Gastric pH

The resistance assessment for gastric pH was carried out based on Kim *et al.* [16] method with modifications. This assay used two types of media, including MRS broth with addition of 37% HCl to achieve a pH of 2.5 and MRS broth without addition of HCl to maintain a pH of 6.8 (as a control). The medium was sterilized at 121 °C for 15 min using autoclave. Moreover, 5 ml of MRS broth-HCl were added to 0.5 ml of the bacterial isolate and incubated at 37 °C for 3 and 6 h. Then, absorbance was read at 600 nm. This was carried out three times. Resistance was expressed as a percentage. According to [17], percentage of the resistance of LAB isolates can be calculated using the following formula:

$$Durability\ (\%) = \frac{\text{Growth of LAB isolates at pH 2.5}}{\text{Growth of LAB isolates at pH 6.8}} \times 100$$

2) Resistance of Rinuak Fish (Psilopsis sp.) from Lake Maninjau, West Sumatra, Indonesia, to Bile Salts

The resistance assessment of bile salt was carried out based on a method from Gotcheva V *et al.* [18] with modifications. Various concentrations of bile salt of 0, 0.3 and 0.5% were added to the MRS broth media. The media were autoclaved at 121 °C for 15 min. The bacterial isolate (0.5 ml) was transferred into 5 ml of MRS broth added with 0, 0.3 and 0.5% ox-gall and incubated at 37 °C for 5 h. The MRS broth containing no bile salt was set as the control and compared with the treatments. The LAB growth was assessed using UV spectrophotometry based on the absorbance at 600 nm. All assessments were carried out in three replications. Isolate resistance was expressed as a percentage. According to [19], percentage of the resistance of LAB isolates can be calculated using the following formula:

$$Durability\ (\%) = \frac{\text{LAB isolate growth at 0.3 or 0.5\%}}{\text{LAB isolate growth at 0\%}} \times 100$$

g. Screening of Lactic Acid Bacteria from Rinuak Fish (Psilopsis sp.) in Lake Maninjau for their Antimicrobial Potentials

The lactic acid bacteria antimicrobial activity assay

Three pathogens of *Salmonella* typhi, *Staphylococcus aureu*s and *Escherichia coli* were assessed for antimicrobial activity of LAB using modified paper diffusion method [20]. Briefly, 1 ml of LAB culture enriched for 48 h was collected using micropipette and transferred into a sterile microtube. This was centrifuged at 10,000 rpm for 5 min and the supernatant was collected for antimicrobial resistance assessment. Then, 0.4 g of NA media was homogenized by heating using hot plate and sterilizing using autoclave. Then, 40 µl of the bacterial isolate enriched for 24 h were poured into a Petri dish containing 20 ml of NA media and cooled down until the media hardened. The paper disk was soaked in LAB isolate suspension for approximately 10 min. After the agar solidified, the paper disk was set on NA media, which contained isolates of pathogenic bacteria. Then, positive control was set by dropping LAB supernatant (20 µl) onto sterile test papers, including 10 g of penicillin, 30 g of kanamycin and 10 g of ampicillin. This was incubated at 37 °C for 24 h anaerobically. After 24 h, diameter of the inhibition zone was reported using caliper [21].

h. Antimicrobial Assay of Crude Bacteriocin Supernatant

Briefly, 1 ml was cultured in 9 ml of MRS solution at 37 °C and incubated for 2 d. This was centrifuged at 14,000 rpm for 5 min. Then, 0.22-µl membrane filter was used to filter the supernatant. To eliminate the barrier effect because of the presence of organic acids, 1 N NaOH solution was added to the cell-free supernatant to maintain pH 6.5 [21]. The bacterial pathogens were grown aerobically at 37 °C for 24 h. Then, 0.2% pathogenic bacteria were transferred onto 20 ml of MHA solution at 50 °C. After the gelatin was solid, a 6-mm well was made in the media using cork borer. Furthermore, 50 µl of supernatant were transferred into the wells and set 10–15 min. The incubation was carried out at 37 °C for 24 h under aerobic conditions. Antimicrobial activity of bacteriocins in the supernatant was verified by varying the time intervals of 15, 30 and 60 min at 100 °C. The supernatant of LAB was investigated for its ability in inhibiting bacteria, including *E. coli* O157, *S. aureus* ATCC 25923 and *Salmonella* enteritidis ATCC 13076 via similar methods. The inhibition zone was associated with existence of bacteriocin compounds and its dimension was recorded using caliper [22].

i. Isolation and Characterization of 16S rRNA

Genome isolation was carried out using genomic DNA mini kit. Lysozyme was used at a concentration of 20 mg ml^{-1} to lyse the bacterial cell walls. The 16S rRNA gene was amplified using selected bacterial genomic DNA kit. Amplification was carried out using reverse primer 1387R (5'-GGGCGGGGTGTACAAGGC-3') and forward primer 63F (5'-CAGGCCTAACACATGCAAGTC-3'). Reactions were carried out in a total volume of 50 µl. The PCR mixture contained 25 µl of DreamTaq Green DNA polymerase (Thermo Fisher Scientific, USA), 22 µl of milli Q water (MQ), 1 µl of the template and 1 µl of each forward or reverse primers (10 µM each, IDT synthesis). Amplification conditions included preheating at 95 °C for 5 min and then denaturation at 95 °C for 30 s, primer annealing at 58 °C for 30 s, 1 min extension step at 72 °C for 35 cycles and post

cycling extension for 5 min at 72 °C. The reaction was carried out in a thermal cycler (Biometra T-Personal Thermal Cycler, USA). The amplified DNA was added to GelRed nucleic acid gel stain and electrophoresed on agarose gels [1.5% (w/v) in TBE buffer] at 100 V for 60 min. The amplicons could be visualized using gel documentation imager. The PCR amplification product was purified using absolute ethanol Na-acetate method and then sequenced [23].

j. The BLAST and Phylogenetic Analysis

Phylogenetic analysis method was carried out based on former studies [24]. Sequence data were collected using BioEdit software and then converted to FASTA format. The BLAST was used to carry out sequence analysis (http://www.ncbi.nl-m.nih.gov/blast/cgi). Moreover, DNA sequences were imported into Clustal W2 (http://www.ebi.ac.uk/Tools/clustalw2/) to carry out phylogenetic analysis. A phylogenetic tree was created using BLAST MEGA v.6.0 (http://www.megasoftware.net) and neighbor-joining (NJ) method.

k. Data Analysis

Data were present as mean ±SD (standard deviation). Statistical significance was reported using one-way analysis of variance (ANOVA) and SPSS software v.26 (IBM, USA). Tukey post-hoc test was used to assess significant differences between group means, with a *p*-value of < 0.05 as statistically significant.

3. Results and Discussion

a. Isolation of Lactic Acid Bacteria in Rinuak Fish (Psilopsis sp.) from Lake Maninjau, West Sumatra, Indonesia

The LAB selective media (MRS broth and agar), providing appropriate nutrients and pH for LAB growth, were used in the serial dilution-agar plate method. This allowed LAB to grow and reproduce effectively. Serial dilution was necessary to decrease bacterial density, ensuring individual colonies grow separately instead of clustering together. The MRS broth and agar as the sources of nutrition and appropriate pH for LAB growth were used in serial dilution-agar plates to isolate LAB. Serial dilution was needed to decrease density of the inoculated LAB to enable the LAB to develop in colonies independently of one another, rather than in piles [19] (Figure 3). Single colonies that were round, convex, yellowish-white and shiny grew separately with various diameter sizes. These were re-inoculated on MRS agar using streak method to achieve pure isolates of LAB from rinuak fish. These findings were similar to those of Purwati study, which showed colonies of LAB on MRS agar as yellowish-white colonies. Details on the isolation and purification of 15 LAB isolates were as follow: three isolates of IR1 (IR1.1, IR1.2 and IR1.3), five isolates of IR2 (IR2.1, IR2.2, IR2.3, IR2.4 and IR2.5), one isolate of IR3 (IR3) and six isolates of IR4 (IR4.1, IR4.2, IR4.3, IR4.4, IR4.5 and IR4.6). A higher number of LAB isolates (n = 15) was achieved in this study, compared to previous reports, which achieved 9–12 isolates in Tilapia fish [25] and 10–13 isolates in Bilih fish [24].

The observed differences in the diversity of LAB types within the samples might indeed be affected by the environmental conditions at the respective sampling locations. For example, Sample IR.1 was collected from Maninjau Village, an area with high population density and a traditional market, while IR.3 originated from Duo Koto Village, a well-known tourist destination. Such anthropogenic activities in these areas might contribute to pollution of the close lake ecosystem, including household and market waste directly discharged into Lake Maninjau. In contrast, IR.2 and IR.4 were collected from relatively undisturbed mid-lake regions—Tanjung Sani Village and Koto Malintang Village respectively—where the water depth exceeded 100 m and minimal human activity was observed, potentially preserving a further appropriate aquatic environment.

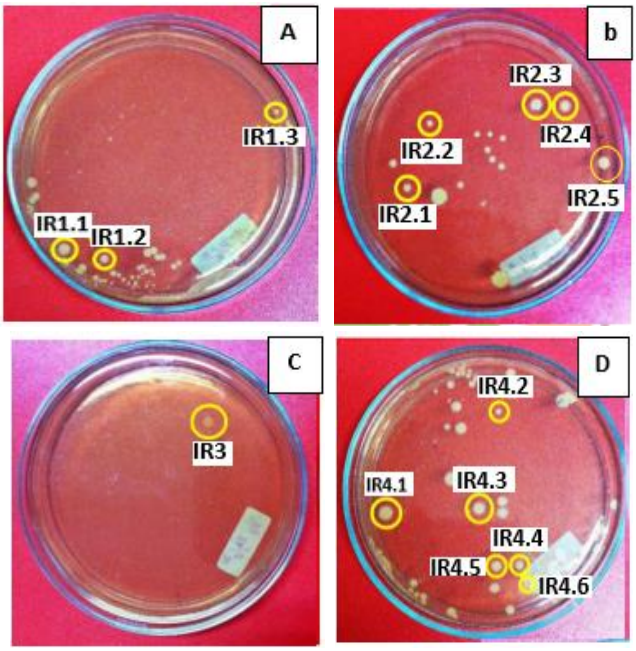

Figure 3. Lactic acid bacterial isolates from four Lake Maninjau rinuak fish samples using serial dilution-agar plate method. The single colony that characterized the isolates was chosen as LAB-Rinuak Fish. Panels A to D showing three isolates of IR.1, five isolates of IR.2, one isolate of IR.3 and six isolates of IR.4, respectively

However, it is acknowledged that geographic origin alone might not fully responsible for the variations in LAB diversity. Several other ecological and biological factors such as the natural diet of rinuak fish, differences in water temperature, seasonal fluctuations and post-capture handling practices (e.g. time to processing or storage conditions) could play significant roles in shaping the

microbiota. These variables might affect selective pressures and survival conditions for specific LAB strains. Further studies incorporating water physicochemical analyses, fish diet profiling and standardized handling procedures are recommended to strengthen the interpretation of LAB diversity patterns. A further holistic approach is necessary to elucidate the multifactorial nature of LAB colonization and persistence in rinuak fish across various environments. Survival of microorganisms depends greatly on environmental conditions and is affected by their food sources [26].

b. Characterization of Lactic Acid Bacteria in Rinuak Fish (Psilopsis sp.) from Lake Maninjau, West Sumatra, Indonesia

The morphological characteristics of all isolates were carried out microscopically through Gram staining (Table 2). The LAB is classified as Gram-positive bacteria that can bind crystal violet-iodine complexes and preserve the purple color of their cells [27]. The stages in Gram staining are crystal violet as the primary dye, Lugol solution (KI-I2) as the mordant, 96% alcohol as the decolorizing agent and safranin solution as the counterstain. Crystal violet dissociates in solution into CV^+ and Cl^-. These ions then penetrate the bacterial membranes and cell walls. Moreover, CV^+ ions react with negatively charged bacterial cell components and make bacterial cells (-) react with CV+ purple. Addition of iodine solution (I^- or I_3) forms a crystal violet-iodine complex (CV-I) in the inner and outer layers of the cells; thereby, strengthening purple color of the bacterial cells [28]. The cell walls of Gram-positive bacteria are in the form of thick fibers consisting of 50–90% peptidoglycan. Therefore when decolorizing, cells are dehydrated and purple because the CV-I complex is trapped in their walls. When safranin solution is added, there is no color change in the bacterial cells [29]. Totally, 15 LAB isolates were assessed. All isolates were Gram-positive, indicated by their purple color in Gram staining. Of them, ten isolates were rod-shaped (bacilli) and five isolates were spherical (cocci) (Figure 4).

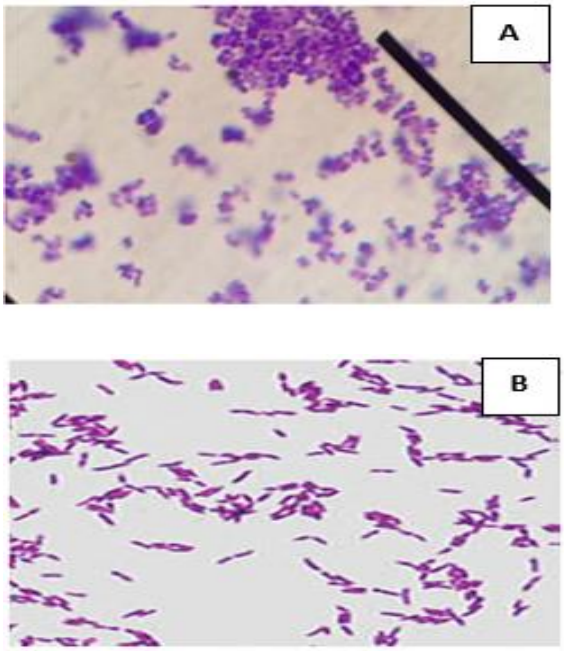

Figure 4. Results of Gram staining of IR.2 (A, cocci-shaped cells) and IR.3 (B, bacilli-shaped cells) isolates. Purple cells indicating Gram-positive bacteria

Table 2. Morphological and biochemical characteristics of 15 isolates of rinuak fish (*Psilopsis* sp.) from Lake Maninjau, West Sumatra, Indonesia

BAL isolate	Microscopic Characteristics		Biochemical Characteristics	
	Gram Staining Test	Cell Shape	Catalase Test	Fermentation Type Test
IR1.1	Positive	Cocci	Negative	Heterofermentative
IR1.2	Positive	Cocci	Negative	Heterofermentative
IR1.3	Positive	Cocci	Negative	Heterofermentative
IR2.1	Positive	Basil	Negative	Homofermentative
IR2.2	Positive	Basil	Negative	Homofermentative
IR2.3	Positive	Basil	Negative	Homofermentative
IR2.4	Positive	Basil	Negative	Homofermentative
IR2.5	Positive	Basil	Negative	Homofermentative
IR3	Positive	Cocci	Negative	Heterofermentative
IR4.1	Positive	Basil	Negative	Homofermentative
IR4.2	Positive	Basil	Negative	Homofermentative
IR4.3	Positive	Basil	Negative	Homofermentative
IR4.4	Positive	Basil	Negative	Homofermentative
IR4.5	Positive	Basil	Negative	Homofermentative
IR4.6	Positive	Basil	Negative	Homofermentative

Another biochemical characteristic of LAB was through fermentation-type testing (Table 2). Lactate-producing bacteria can be homofermentative or heterofermentative depend on the primary fermentation product. Production of lactic acid indicates that LAB is homofermentative. Furthermore, heterofermentative LAB in addition to produce lactic acid can produce ethanol, CO_2 and acetic acid [17]. This type of fermentation test is carried out using gas bubbles form in the Durham tube, which is placed upside down in the LAB liquid culture after incubation for 48 h. The LAB is addressed as heterofermentative if gas is detected in the Durham tube. In contrast, LAB is disclosed as homofermentative if the Durham tube is empty of gas [23]. In this study, 11 LAB isolates from rinuak fish were homofermentative, three LAB isolates from IR1 and one LAB isolate from IR3 were heterofermentative. This was due to the long storage time of fermentation and IR1 and IR3 were located in densely populated areas, markets and tourist attractions. High population density, existence of traditional markets and tourist attractions at the sampling location might damage rinuak fish ecosystem because waste from homes and markets is thrown directly into Lake Maninjau. The environment includes a significant effect on the lives of microorganism conditions [26].

c. Selection of Lactic Acid Bacteria in Rinuak Fish (Psilopsis sp.) from Lake Maninjau, West Sumatra, Indonesia, as Probiotic Candidates

The LAB selection as probiotic candidates was carried out on ten LAB isolates, which were classified as Gram-positive bacteria, did not produce catalase enzyme and ethanol in the fermentation type test, classified as homofermentative.

d. Resistance of Lactic Acid Bacteria in Rinuak Fish (Psilopsis sp.) from Lake Maninjau, West Sumatra, Indonesia, to Gastric pH

The LAB resistance to gastric pH assay was carried out at pH 2.5 because the pH in the gizzard and proventriculus was 2.5–3.5. The feed transit time was 70 min and assessed for 3 and 6 h. The resistance of LAB isolates was assessed at 600 nm using spectrophotometer (Table 3) [17].

Data in Table 3 show that all LAB isolates include ability to survive at pH 2.5 for 3 and 6 h of incubation time. Including a minimum resistance of 50% indicate that all LAB isolate are probiotics potential. These findings were similar with those that probiotic LAB should show a survival rate of ≥ 50% at low pH, as suggested by [14]. Ten LAB isolates were assessed for resistance to pH 2.5 through spectrophotometry at 600 nm. Isolates that showed the highest resistance to low pH conditions included IR2.2, IR2.4, IR4.1, IR4.3 and IR4.5. All of these isolates were achieved from fermented rinuak fish from Lake Maninjau. Their survival rates after 3 h of incubation at low pH included 88.01, 84.16, 84.70, 86.64 and 84.24%, respectively. After 6 h of incubation, a slight decrease in resistance was observed with survival rates of 84.96, 80.83, 80.88, 82.72 and 82.16%, respectively. These results indicated that the selected LAB isolates included high tolerance to acidic conditions over time, which is a key criterion for probiotic selection. This is according to [15], stating that the greatest survival of LAB isolates as probiotics are isolates that include small decreasing difference. This study produced better outcomes than previously studies that isolated LAB from okara, which included 74.02% of survival rate for 2 h at pH 2.5 [24]. In previous study [17], LAB strains of *Lactobacillus brevis*, *L. plantarum* and *Pediococcus indurans* isolated from traditional pickles survived at pH 2.5 for 4 h of 33–64, 35–85 and 40–76%, respectively [16]. The survival rate of *L. fermentum* strains isolated from fermented milled flour was ≥ 80% after incubation for 4 h at pH 2.5 [14]. The LAB of probiotic candidates must be able to endure the exorbitant conditions of the gastrointestinal tract (GIT) from the mouth to the intestines and then live in colonies on the surface of the intestines and the acidity of the stomach functions as the first gate for selecting microbes prior to passing into the intestines [23].

Table 3. Resistance of lactic acid bacteria in rinuak fish (*Psilopsis* sp.) from Lake Maninjau, West Sumatra, Indonesia, to gastric pH

Number	LAB isolate	Time (3 hours) (%)	Time (6 hours) (%)
1	IR2.1	74,83 ± 5.47	46,45 ± 1.77
2	IR2.2	88.01 ± 3.76	84.96 ± 2.09
3	IR2.3	81,18 ± 2.68	62,98 ± 2.59
4	IR2.4	84.16 ± 2.09	80.83 ± 2.71
5	IR2.5	74.57 ± 3.42	59.79 ± 1.75
6	IR4.1	84,70 ± 4.52	80,88 ± 0.99
7	IR4.2	74.57 ± 3.42	59.79 ± 1.75
8	IR4.3	86,64 ± 2.52	82,72 ± 5.51
9	IR4.4	80,64 ± 2.52	79,72 ± 5.51
10	IR4.5	84,24 ± 2.94	82,16 ± 1.36

Note: Values are expressed as mean ± standard deviation; n=10

e. Resistance of Lactic Acid Bacteria in Rinuak Fish (Psilopsis sp.) from Lake Maninjau, West Sumatra, Indonesia, to Bile Salts

The resistance of LAB-Rinuak Fish to bile salts was assessed using spectrophotometry at 600 nm with a concentration of 0.3 and 0.5% and incubated for 5 h. Results are listed in Table 4.

Table 4. Resistance of lactic acid bacteria in rinuak fish (*Psilopsis* sp.) from Lake Maninjau, West Sumatra, Indonesia, to bile salts

Number	ILAB isolates	Resistance (%)	
		Bile Salts 0.3%	Bile Salts 0.5%
1	IR2.1	47,22 ± 1.11	34,75 ± 1.35
2	IR2.2	55,41 ± 1.19	51,61 ± 0.52
3	IR2.3	48,69 ± 2.00	36,25 ± 7.77
4	IR2.4	57,35 ± 1.24	52,67 ± 1.19
5	IR2.5	47,57 ± 2.25	38,51 ± 2.61
6	IR4.1	53,49 ± 2.10	50,50 ± 1.47
7	IR4.2	46,65 ± 0.42	37,10 ± 1.02
8	IR4.3	53,24 ± 1.49	51,11 ± 1.46
9	IR4.4	39,86 ± 0.81	28,83 ± 1.24
10	IR4.5	50,37 ± 1.55	47,36 ± 0.74

Note: Values are expressed as mean ± standard deviation; n=10

Findings in Table 4 show that all LAB isolates could survive against bile salts with resistance > 20% under the statement [37]. The major problems for the survival rate of the probiotics selected strains were gastric acidity and bile salts, which at least included 20–40%. According to [38], isolate is verified as a strong probiotic candidate when its survival rate is greater than 50% under low pH conditions, including bile salt resistance. Thus, the LAB isolates that included good probiotic criteria out of 11 isolates included five isolates with ≥ 50% resistance at a concentration of 0.3% bile salts. These included IR2.2, IR2.4, IR4.1, IR4.3 and IR4.5 isolates with resistance at 0.3% bile salt concentration of respectively 55.41, 57.35, 53.49, 53.24 and 50.37%. Resistance decreased when the bile salt concentration (ox gall) increased to 0.5% as respectively 51.61, 52.67, 50.50, 51.11 and 47.36%. The decrease differences of IR2.2, IR2.4, IR4.1, IR4.3 and IR4.5 respectively included 3.80, 4.68, 2.99, 2.14 and 3.01. A slight decrease indicated a large survival rate. This is based on the fact [19] that isolates with a minor decrease difference include the highest opportunity of surviving as probiotic LAB isolates. Characteristics of the isolates included the potentials as probiotics because they were resistant to small intestine bile salts; hence, they could survive in the large intestine. According to [12], LAB could survive in bile salts because it included bile salt hydrolase (BSH) enzyme with the mechanism that the major component of bile salts included bile acids and these bile acids primarily targeted the bacterial membrane. Based on the two highlighted criteria of resistance to gastric pH and tolerated 0.3% bile salts, the other assessment was carried out on five selected isolates that included high ability for the antimicrobial potential assessment.

f. Antimicrobial Activity Assessment of Five Selected Lactic Acid Bacteria Isolates of Rinuak Fish (Psilopsis sp.) from Lake Maninjau, West Sumatra, Indonesia, against Pathogenic Bacteria

Inhibition activity of the selected LAB from Rinuak Fish (*Psilopsis* sp.) to the growth and development of the pathogenic bacteria of *E. coli* ATCC O157, *S. aureus* ATCC 25923 and *Salmonella* enteritidis ATCC 13076 are shown in Table 5.

Results from the study can be seen in Table 5, showing that the clear/inhibitory zone area for each isolate varied greatly, this was because the abilities of each bacteria from each isolate varied. Clear zone was formed due to the metabolism of LAB that produced lactic acid, bacteriocins and hydrogen peroxide, all of which included ability against microbes. The LAB isolates having the largest zone of inhibition for *E. coli* O157 belonged to IR4.3 with 14.46 mm in diameter, while the smallest clear zone diameter was present by IR4.1 with 12.43 mm in diameter. The largest zone of inhibition was detected in IR4.3 with 14.40 mm in diameter against *S. aureus* ATCC 25923 bacteria. In contrast, IR2.4 generated a 12.89-mm zone in diameter, accounted as the smallest zone of inhibition. Against *Salmonella* enteritidis ATCC 13076, the biggest clear zone of inhibition was present by IR4.3 with a diameter of 16.60 mm; whereas, the least zone was produced by IR2.4 with a diameter of 15.64 mm.

Table 5. The antimicrobial inhibitory activity assessment (mm)

LAB isolates	Clear zone diameter (mm)		
	Escherichia coli ATCC O157	*S. aureus* ATCC 25923	*salmonella enteritidis* ATCC 13076
IR2.2	14.06± 0,26	14.53± 0.25	15.23± 0.14
IR2.4	12.79± 0.15	12.89± 0.17	14.64± 0.23
IR4.1	12.43± 0.10	12.95± 0.13	15.84± 0.41
IR4.3	14.46± 0.44	15.01± 0.64	16.60± 0.86
IR4.5	14.28± 0.32	13.85± 0.20	15.68± 0.71

Note: Values are expressed as mean ± standard deviation; n=5

Furthermore, LAB IR4.3 was assessed for its antimicrobial activity to inhibit bacterial pathogens using several antibiotics of kanamycin, penicillin and ampicillin as the positive controls. The purpose of using penicillin, kanamycin and ampicillin was because these antibiotics were effective against Gram-positive bacteria. According [10], penicillin is an antibiotic that is able to inhibit cell walls of bacterial synthesis, including those in the beta-lactam group. Ampicillin is a penicillin derivative that includes a broader spectrum, able to inhibit Gram-positive and Gram-negative bacteria as well as the beta-lactam group. Antibiotics were assessed using paper disks that contained the specified concentrations of 30-µg kanamycin, 10-µg ampicillin and 10-µg penicillin. To assess resistance and sensitivity of bacterial pathogens, pathogens were used using positive-control antibiotics. The clear zone results are shown in Table 6.

Based on Table 6, LAB isolates IR4.3 was able to inhibit the three bacterial pathogens of *E. coli* O157:H7, *S. aureus* ATCC 25923 and *Salmonella* enteritidis ATCC 13076 with diameters of 22.46, 19.34 and 9.41 mm, respectively. The LAB isolate tolerated the three antibiotics, including ampicillin (10 µg), kanamycin (30 µg) and penicillin (10 µg), against *E. coli* O157:H7 (Figure 5).

Overally, Figure 5 illustrates that the LAB IR4.3 from the staining included antimicrobial activity against all the pathogens, compared to penicillin, which did not include inhibition activity to the growth of *Salmonella* enteritidis ATCC 13076 and *S. aureus* ATCC 25923, as well as kanamycin that did not include antimicrobial activity against all the pathogenic bacteria. This was based on a study [21], where the penicillin test bacteria could not inhibit the growth of *E. coli* ATCC O157; thus, it could be reported that *E. coli* ATCC O157 were resistant to penicillins. Results clearly showed that the inhibition zone formed by the LAB IR4.3 included high antimicrobial activity against the pathogenic bacteria. Results achieved were based on an opinion [42], stating that four categories of inhibition zones were reported as very strong (> 20–30 mm), strong (>10–20 mm), moderate (5–10 mm) and weak (<5 mm) inhibition zones. Based on these four categories, the LAB IR4.3, which was isolated from fermented rinuak fish (*Psilopsis* sp.) including a very high clear zones, was categorized as a very strong antibacterial agent against the three bacteria. The capacity of LAB to inhibit the growth of enteric pathogens that live in the digestive system is a critical factor in the selection of these isolates for use as probiotic agents. Another need that probiotic bacteria must include is the ability to produce antimicrobial substances [13].

Table 6. Clear zone diameter of antimicrobial activity assessment of LAB IR4.3 from rinuak fish (*Psilopsis* sp.) from Lake Maninjau, West Sumatra, Indonesia, with antibiotics as positive controls

Sample Code	Clear zone diameter (mm)		
	Escherichia coli ATCC O157	*Staphylococcus aureus* ATCC 25923	*Salmonella enteritidis* ATCC 13076
IR4.3	14.46 ± 0.04	15.01 ± 0.64	16.60 ± 0.86
Penicillin 10 µg	3.80 ± 0.04	-	-
Ampicillin 10 µg Kanamycin	13.28 ± 0.07	18.57 ± 0.03	-
30µg	15.31 ± 0.09	16.17 ± 0.07	7.05 ±0.09

Note: Values are expressed as mean ± standard deviation; n=4

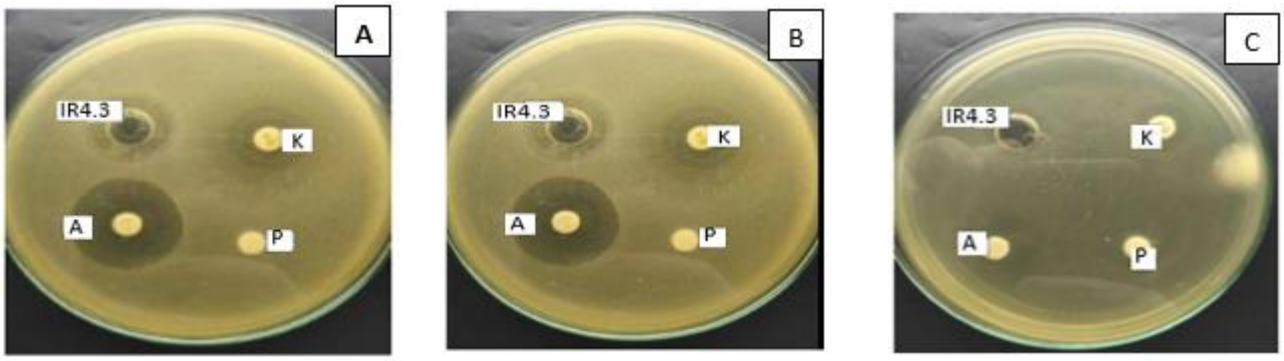

Figure 5. The IR4 antimicrobial activity assessment. (A) Formation of a clear zone against *Escherichia coli* ATCC 0157, (B) formation of a clear zone against *S. aureus* ATCC 25923 and (C) formation of a clear zone against *Salmonella* enteritidis ATCC 13076

g. Antimicrobial Activity of the Crude Bacteriocin Supernatant

After LAB supernatant pH neutralization, antimicrobial activity of the crude supernatant of LAB IR4.3 bacteriocin was assessed. Therefore, the organic acid antimicrobial characteristics were not detected. The major component of antimicrobial compounds in LAB contains components of organic acids, particularly lactic acid [14]. The neutralized supernatant antibacterial efficacy against pathogenic microorganisms is shown in Table 7.

Findings are shown in Table 7 and Figure 6, which demonstrate the antimicrobial efficacy against *E. coli* ATCC O157 of 18.29 mm and *S. aureus* ATCC 25923 of 20.63 mm (Figure 6), following the neutralization of LAB supernatant pH. However, activity of the antimicrobials is not indicated for *Salmonella* enteritidis ATCC 13076.

Results were more important, compared with those previously carried out [8], reporting that LAB isolates from Bilih fish of Lake Singkarak included gross bacteriocin activity against *S. aureus* ATCC 25923 (13.1 mm) and *E. coli* O157:H7 (12.7 mm). Moreover, studies [25] did not find antimicrobial activity in LAB isolates from raw beef after the supernatant pH was neutralized because LAB produced organic acids, which affected antimicrobial activity. Furthermore, another study [16] reported *L. Plantarum* NS from isolates of various fermented food products from freshwater fish, showing that organic acids included a relationship with antimicrobial activity. It was reported that in the final phase of exponential growth (incubation time of 12–15 h), antimicrobial activity was achieved against the pathogenic bacteria of *E. coli, L. monocytogenes* and *B. cereus*. At incubation times of 21 and 24 h, it was reported that *L. Plantarum* NS included the best antimicrobial characteristics against *S. aureus* and *S. typhimurium* ATCC 14028. Furthermore, several results showed that *L. brevis* and *L. plantarum* isolated from fermented bekasam food from South Sumatra included antimicrobial characteristics in supernatants of cell-free culture and were effective to inhibit *E. coli* and *L. monocytogenes* [27]. There are several strategies to prevent bacteriocins from destroying target cells [28], the mechanism for inhibiting the synthesis of lipid II (precursor of cell wall) is stabilizing formation of target membrane pores and inhibiting cell wall biosynthesis. Phospholipid layer binds fatty acids to the positive end of the peptide, showing that the peptide is attached to a target cell membrane. This mechanism brings peptides to membrane-like monomers to form bonds, producing separation leading to the formation of pores and ultimately cell death [29]. Bacteriocins are complex proteins that include bactericidal abilities, especially against Gram-positive bacteria and close species [20].

Table 7. Antimicrobial activity of the crude bacteriocin supernatant from IR4.3 origin of rinuak fish (*Psilopsis* sp.) from Lake Maninjau, West Sumatra, Indonesia

Pathogenic Bacteria	Diameter of Clear Zone (mm)
E. coli ATCC *O157*	18.29 ± 0.03
S. aureus ATCC *25923*	20.63 ± 0.01
Salmonella enteritidis ATCC 13076	-

Note: Values are expressed as mean ± standard deviation; n=3

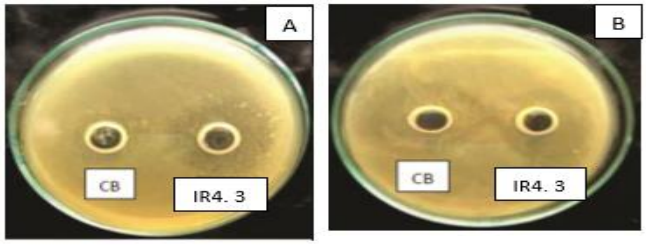

Figure 6. Antimicrobial activity of the crude bacteriocins after pH neutralization (CB) and prior to pH neutralization (IR4.3) against *Escherichia coli* ATCC O157 (A) and *Staphylococcus aureus* ATCC 25923 (B)

h. Identification of the Selected IR4.3 Isolates of Rinuak Fish (Psilopsis sp.) Using 16S rRNA

Results of the amplification of 16S rRNA gene using polymerase chain reaction

Findings of electrophoresis indicated that the bacterial 16S rRNA gene from the isolation of rinuak fish (*Psilopsis* sp.) LAB was successfully amplified using PCR. This was demonstrated with a 1,500-bp PCR product fragments, using primer 27F (AGAGTTGATCCTGGCTGAG) and primer 1429 R (GTTTACCTTACGACTT). The LAB 16S rRNA gene amplification results from rinuak fish (*Psilopsis* sp.) were used to ensure that the genomic DNA isolation was successful. To identify LAB, results of 16S rRNA gene amplification were used. Sequencing of the 16S rRNA gene nucleotides was carried out after its amplification. Figure 7 shows results of the electrophoresis of PCR products from LAB isolates. Comparing ribosomal RNA sequences, molecular techniques are used to investigate the genetic links of LAB [24]. Moreover, 16S rRNA can directly be sequenced without the need of PCR amplicon cloning. Results of the DNA amplification of a 1,500-bp fragment are shown in Figure 7. This demonstrated that the particular primers in the study was able to recognize bacteria at the strain level.

Chromatograms of sequencing results from the direction of reading the forward and reverse primers of the 16SrRNA gene from IR4.3 were aligned using SeqMan software. Length of the 16S rRNA gene amplicon amplified with the primers of 16S rRNA_27F and 16S rRNA_1525R was 1,500 bp. Moreover, chromatogram from reading the forward primer of 16SrRNA_27F was 726 bp. Chromatogram from reading the reverse primer of

16SrRNA_1525R was 1,131 bp. As a weakness of the Sanger sequencing method, several bases at the ends of the chromatogram include peaks that overlap and are not clear. Hence, these bases are removed [21]. The chromatogram resulting from reading the forward primer of 16SrRNA_27F at the 5' end was cut with 40-bp long. At a position of nearly 400 bp toward the end of the 3' peak in this chromatogram, it became increasingly sloping and even flat. Bases in the sloping peak were edited by adjusting the base sequence in the reverse primer reading. The chromatogram resulting from reading the reverse primer 16SrRNA_1525R at the 3' end was cut with 81-bp long and at the 5' end with 30-bp long. After the editing procedure, length of the sequence that included in the 16SrRNA gene fragment from IR4.3 was 1500 bp. The edited chromatogram visualization was as follows.

>**Contig_IR4.3**
AAAAGGTTCTCGTATCATTCTGGATGACCTGCGGGCATTAGCTTGTGGTGGGGTAAGGCCTACCAAGGCGATGATGCATAGC
CGAGTTGAGAGACTGATCGGCCACAATGGGACTGAGACACGGCCCATACTCCTACGGGAGGCAGCAGTAGGGATCTTCCAC
AATGGGCGCAAGCCTGATGGAGCAACACCGCGTGAGTGAAGAAGGGTTTCGGCTCGTAAAGCTCTGTTGTTAAAGAAGAAC
ACGTATGAGAGTAACTGTTCATACGTTGACGGTATTTAACCAGAAAGTCACGGCTAACTACGTGCCAGCAGCCGCGGTAAT
ACGTAGGTGGCAAGCGTTATCCGGATTTATTGGGCGTAAAGAGAGTGCAGGCGGTTTTCTAAGTCTGATGTGAAAGCCTTCG
GCTTAACCGGAGAAGTGCATCGGAAACTGGATAACTTGAGTGCAGAAGAGGGTAGTGGAACTCCATGTGTAGCGGTGGAAT
GCGTAGATATATGGAAGAACACCAGTGGCGAAGGCGGCTACCTGGTCTGCAACTGACGCTGAGACTCGAAAGCATGGGTAG
CGAACAGGATTAGATACCCTGGTAGTCCATGCCGTAAACGATGAGTGCTAGGTGTTGGAGGGTTTCCGCCCTTCAGTGCCGG
AGCTAACGCATTAAGCACTCCGCCTGGGGAGTACGACCGCAAGGTTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGC
GGTGGAGCATGTGGTTTAATTCGAAGCTACGCGAAGAACCTTACCAGGTCTTGACATCTTGCGCCAACCCTAGAGATAGGGC
GTTTCCTTCGGGAACGCAATGACAGGTGGTGCATGGTCGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACG
AGCGCAACCCTTGTTACTAGTTGCCAGCATTAATTGGGCACTCTAGTGAGACTGCCGGTGACAACCGGAGGAAGGTGGGGA
CGACTCAGATCATCATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGGACGGTACAACGAGTCGCGAACTCGCGAG
GGCAAGCAAATCTCTTAAAACCGTTCTCAGTTCGGACTGCAGGCTGCAACTCGCCTGCACGAAGTCGGAATCGCTAGTAATC
GCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGAGAGTTTGTAACACCCAAA
GTCGGTGGGGTAACCTTTAGGAGCCAGCCGCCTAAGGGGACAATGG

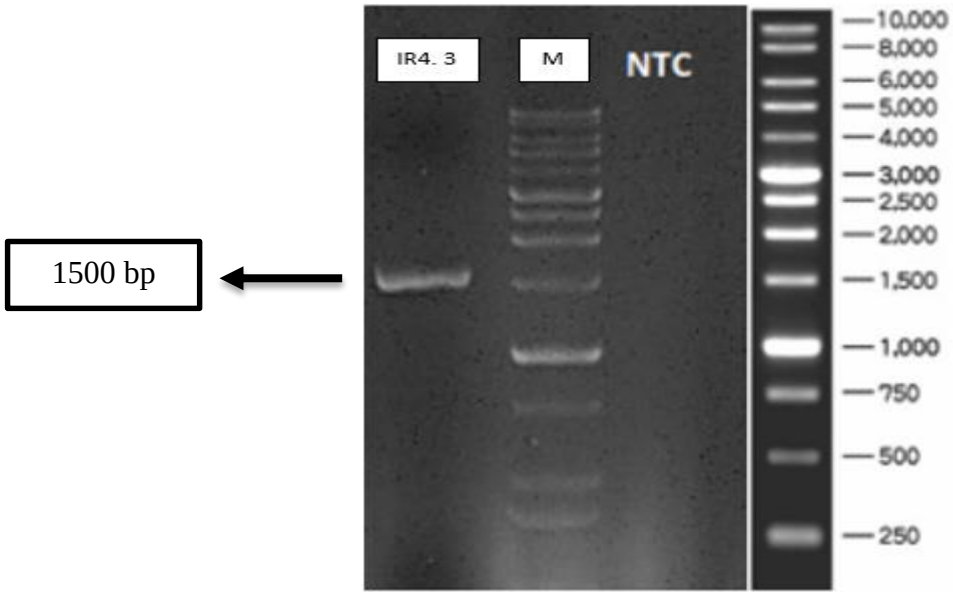

Figure 7. Results of the amplicon electrophoresis of IR4.3 origin from rinuak fish (*Psilopsis* sp.) [M = Marker, A = LAB IR4.3 origins of rinuak fish (*Psilopsis* sp.)]

Of 250 sequence data from BLAST results for isolating IR4.3 strain, a 100% query cover value was achieved. Then, the identity value percentage was achieved, ranging 99.21–99.64%. Totally, 15 *L. Fermentum* bacterial sequence data in GenBank were selected for further use in genetic relationship analysis. Information characteristics of the 15 bacterial sequence data were as follows.

Based on Table 8, IR4.3 isolate included similarity of 99.64% to the genome sequence and partial strains of *Lactobacillus*. Based on a fact that a sequence is homologous if its resemblance to another sequence is greater than 99 %, the MEGA tool was used to visualize the results of the BLAST after initial use of BioEdit v7.0. Based on the phylogenetic visualization results, the fermented fish sample isolated from rinuak fish (*Psilopsis* sp.) in the study

showed an apomorphic relationship with *L. fermentum*. Previous studies stated that the characters were apomorphic and plesiomorphic [22]. Apomorphic characters are characters that change and inherited, while synapomorphic characters are inherited characters detected in monophyletic groups [23]. Regarding the query cover value, 15 species were selected from the BLAST results to generate the phylogenetic tree. Then, FASTA format for the top 15 species were retrieved to create a phylogenetic tree.

i. Phylogenetic Tree

Based on the BLAST results, a phylogenetic tree was created to include the level of relationship between the isolates and other species using NCBI. Creating a phylogenetic tree via the Mega X, FASTA files. The downloaded files were aligned first and then a phylogenetic tree was created. Neighbor-joining method was used to construct the phylogenetic tree with a bootstrap value of 1,000. Kimura 2-parameter method was used to analyze evolutionary distances. Phylogenetic tree analysis demonstrated relationships between the IR4.3 isolate and 15 others reference bacteria from the GenBank based on the 16SrRNA gene fragment sequence. In the BLAST results, IR4.3 was closely linked to *Lactobacillus* genus with 100% similarity as shown in Figure 8. Results were analyzed because it was suspected that this isolate belonged to a similar genus and could produce antimicrobials. The phylogenetic tree illustrating the relationship between IR4.3 isolate and 15 other reference bacteria from the GenBank based on the 16S rRNA gene fragment sequence was as follows:

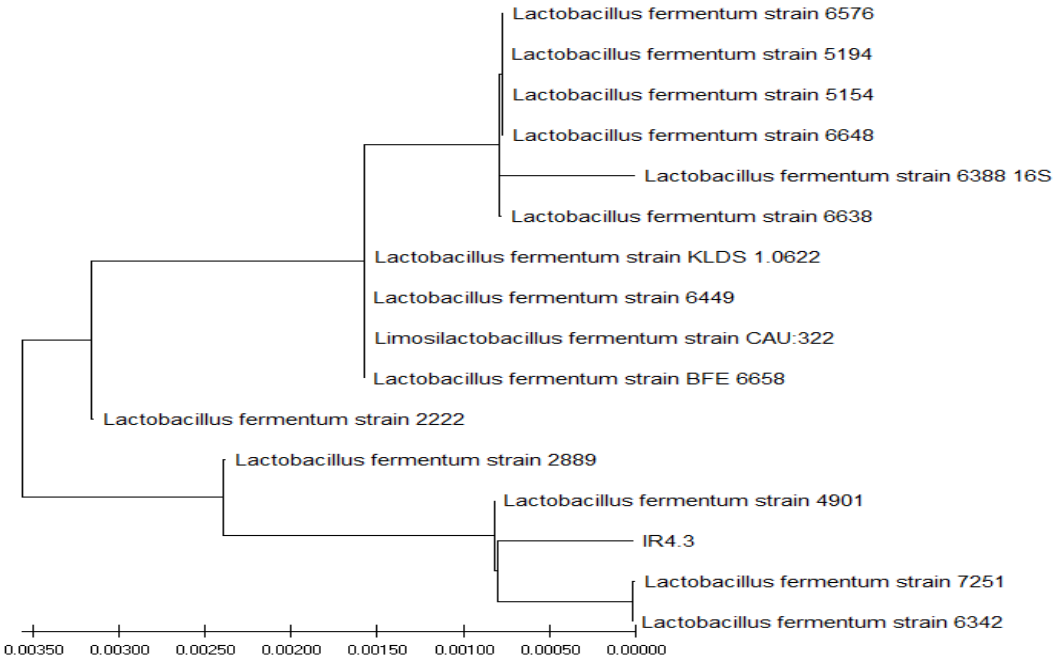

Figure 8. Phylogenetic tree of IR4.3 isolate

Table 8. The BLAST results of rinuak IR4.3 (*Psilopsis* sp.) isolate from Lake Maninjau, West Sumatra, Indonesia

Number	Description of *Lactobacillus* Bacteria strains	Query cover (%)	Accession number	Percent identification (%)
1	*Lactobacillus Fermentum* Strain 6576		MT463820.1	
2	*Lactobacillus Fermentum* Strain 5194		MT463446.1	
3	*Lactobacillus Fermentum* Strain 5154		MT510246.1	
4	*Lactobacillus Fermentum* Strain 6648		MT463855.1	
5	*Lactobacillus Fermentum* Strain 6388		MT463793.1	
6	*Lactobacillus Fermentum* Strain 6638		MT463846.1	
7	*Lactobacillus Fermentum* Strain KLDS 1.0622		EU419596.1	
8	*Lactobacillus Fermentum* Strain 6649	100 %	MT515877.1	99,64 %
9	*Limosilactobacillus fermentum* strain CAU:322		MF369883.1	
10	*Lactobacillus Fermentum* Strain BFE 6658		AY929282.1	
11	*Lactobacillus Fermentum* Strain 2222		MT604718.1	
12	*Lactobacillus Fermentum* Strain 2889		MT611851.1	
13	*Lactobacillus Fermentum* Strain 4901		MT505643.1	
14	*Lactobacillus Fermentum* Strain 7251		MT516047.1	
15	*Lactobacillus Fermentum* Strain 6342		MT463751.1	

Generally, [24] showed that the evolutionary history of microorganisms could be reported using neighbor-joining method. Microorganisms in similar taxa usually cluster together in phylogenetic trees and include better bootstrap values [15]. In this study, a phylogenetic tree was drawn to investigate evolutionary distances using p-distance method. A total of 15 nucleotide sequences and codon positions were recorded using MEGA 7.0, as reported by [26] for evolutionary analysis. A phylogenetic tree (Figure 8) was reconstructed. The constructed phylogenetic tree resulted in two major branches consisting of the 15 bacteria. This occurred because the 16S rRNA gene fragment sequence included a similarity percentage of 100%, as verified by the results of BLAST, alignment and genetic distance calculations. Therefore, identification of IR4.3 isolate could be carried out at the genus level. Hence, it was concluded that IR4.3 isolate included *L. fermentum* strain 4901. These bacteria are homofermentative (only producing lactic acid) and cannot use pentoses (carbohydrates with a C5 atom). In general, [20] has stated that the bacteria of the lactobacilli group are bacteria that are included in the LAB category and these bacteria are widely used as probiotic agents because these bacteria produce the final products of the metabolic process, including lactic acid, which are generated from fermentation. The LAB is an anaerobic bacterium that is generally detected in fermented food and beverage products such as cheese, pickles, kimchi, fish stock and yoghurt. Therefore, it can be concluded that *L. fermentum*, which are classified as *Lactobacillus*, can be used as probiotics that are good for health. Moreover, LAB in fish are bacteria that are halal for consumption because they are originated from rinuak fish (*Psilopsis* sp.), different from several types of LAB used in fermented milk products such as *Bifidobacterium* isolated from baby feces. Technically, [23] has stated that in addition to food security which must be safe with good quality, food products of animal origin must meet the SHWH (safe, healthy, whole and halal) criteria. Additionally, [27] has reported that *L. fermentum* was verified to include characteristics of a probiotic agent that can survive in acidic conditions and inhibit the growth of Gram-positive and Gram-negative bacteria.

The present study varied from that of [28], which detected that the LAB originating from tilapia fish paste were *L. fermentum*. Isolation of LAB from shrimp paste carried out by [29] achieved the species *L. plantarum*. Studies by [30], investigating LAB strains from Sulawesi fermented milkfish Chanos-rice mixture of Burong Bangus reported various strains such as *Enterococcus faecalis*, *Tetragenococcus muriaticus*, *L. delbrueckii* subp. *delbrueckii* and *Carnobacterium divergens*. In a study by [13] on the isolation of LAB from tilapia fish (*Oreochromis niloticus*), LAB species were isolated by sequencing *P. pentosaceus* and *E. avium*. The species of bacteria varied due to the various types of fermented fish, including fresh fish, ingredients for fermentation and method of fermentation. The strain of *L. brevis* WD19 was isolated from the Algerian goat milk [21]. The finding of this study varied significantly from those of [22], who investigated presence of *B. cereus* strain HRV22 from isolation of the genome of freshwater shrimps using 16S rRNA gene sequencing method.

4. Conclusion

Results of DNA isolation and amplification with the 16S rRNA gene and primers of 27F and 1525R showed that the IR4.3 PCR product included 1500 bp and the isolate included the best antimicrobial potential of halal probiotic LAB, compared to 14 other bacterial isolates from rinuak fish in Lake Maninjau, West Sumatra, Indonesia. Isolate IR4.3 from rinuak fish showed 99.64% sequence similarity to *L. fermentum* strain 4901 based on 16S rRNA gene analysis, indicating that it belonged to a similar species. However, further genetic and phenotypic characterizations are needed to assess if IR4.3 represents a novel strain. The isolated species demonstrates potential as candidate probiotic LAB for use in natural food preservation and possibly for supporting treatments of diarrhea and typhoid fever. However, this potential must be verified through further *in vivo* studies and clinical validation. However, advanced studies of bacteriocins produced by LAB from rinuak fish in Lake Maninjau are still needed, including characterization of bacteriocins for food biopreservation.

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6. Declaration of competing interest

The authors report no conflict of interest.

7. Authors' Contributions

Heppy Setya Prima: Writing – Original Draft, Conceptualization, Methodology, Investigation; Rusfidra: Funding Acquisition, Validation; Fatridha Yansen: Writing – Review & Editing, Writing – Original Draft, Data Analysis, Investigation; Fajri Maulana: Conceptualization; Mia Ayu Gusti : Conceptualization

8. Using Artificial Intelligent Chatbots

No AI-assisted technologies were used in the preparation of this manuscript" or "No generative AI technologies or

tools were employed in the writing or preparation of this manuscript

9. Ethical Consideration

Hereby, I am Heppy Setya Prima consciously assure that for the manuscript "Characterization, molecular identification and antimicrobial activity of lactic acid bacteria with potentials as halal probiotics isolated from Rinuak fish (Psilopsis sp.) in Lake Maninjau, West Sumatra, Indonesia" the following is fulfilled: 1) This manuscript represents the authors' original work and has not been published previously in any form. 2) The manuscript is not under consideration for publication by any other journal or publisher. 3) The content of the paper accurately and comprehensively reflects the authors' own research and analysis. 4) All significant contributions made by co-authors and collaborators are properly acknowledged. 5) The findings are appropriately contextualized within the framework of existing and prior research. 6) All sources utilized are properly cited, with any directly quoted material clearly indicated using quotation marks and appropriate referencing. 7) Each author has been actively and substantially involved in the research and preparation of the manuscript and collectively assumes full responsibility for its content.

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خصوصیات، شناسایی مولکولی و فعالیت ضد میکروبی باکتری های اسید لاکتیک با پتانسیل به عنوان پروبیوتیک حلال جدا شده از ماهی رینوآک (جنس سایلوپسیس) در دریاچه مانین‌جاو غرب سوماترا اندونزی

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چکیده

سابقه و هدف: بررسی باکتری‌های اسید لاکتیک (LAB) در ادغام گواهی حلال خاص، یکی از مباحث اصلی تحقیق در زمینه‌های بهداشت، صنایع غذایی، دامداری و کشاورزی است. این مطالعه با هدف بررسی پتانسیل ضدمیکروبی باکتری‌های اسید لاکتیک پروبیوتیک جدا شده از ماهی رینواک (جنس سایلوپسیس) از دریاچه مانینجائو، اندونزی انجام شد.

مواد و روش ها: در مجموع ۱۵ باکتری اسید لاکتیک از چهار نمونه ماهی رینواک (جنس سایلوپسیس) جدا شد و بررسی ویژگی‌های آنها به عنوان کاندیداهای پروبیوتیک با استفاده از تست‌های آزمایشگاهی مرسوم و روش‌های تعیین توالی S rRNA۱۶ ادامه یافت.

یافته‌ها و نتیجه‌گیری: پنج جدایه به عنوان کاندیداهای پروبیوتیک شناسایی شدند، یعنی IR2.2، IR2.4، IR4.1، IR4.3 و IR4.5 به دلیل مقاومت خوب آنها در برابر pH معده از ۸۴/۲۴ تا ۸۸/۰۱ و توانایی بقای آنها در برابر نمک‌های صفراوی (درصد مقاومت؛ ۵۰/۳۷ تا ۵۷/۳۵٪). IR4.3 با قطر هاله عدم رشد به ترتیب ۲۲,۴۶ میلی‌متر، ۱۹,۳۴ میلی‌متر و ۹,۴۱ میلی‌متر، بیشترین فعالیت ضدمیکروبی را علیه *اشریشیا کلی* ATCC 0157، *استافیلوکوکوس اورئوس* ATCC 25923 و *سالمونلا انتریدیس* ATCC 13076 ایجاد کرد. روش تعیین توالی S ۱۶ RRNA تأیید کرد که باکتری اسید لاکتیک جدا شده از ماهی رینواک (جنس سایلوپسیس) ۹۷/۶۹درصد شباهت به سویه *لاکتوباسیلوس فرمنتوم* ۴۹۰۱ دارد که به عنوان عامل ضداسهال و ضدتیفوئید و نگهدارنده طبیعی غذا برای افزودن به محصولات غذایی و دارویی مطابق با حلال مناسب و نویدبخش است.

واژگان کلیدی: نگهدارنده غذا، ماهی بومی، تخمیر میکروبی، توصیف مولکولی، سویه *لاکتوباسیلوس فرمنتوم* ۴۹۰۱