Annals of R.S.C.B., ISSN:1583-6258, Vol. 25, Issue 4, 2021, Pages. 8581 - 8596
Received 05 March 2021; Accepted 01 April 2021.

Isolation, Characterization and Identification of Molecular Lactic Acid is Isolated from Bilih Fish (*Mystacoleucuspadangensis*) Lake Singkarak Potential as a Probiotic

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ABSTRACT

The Singkarak lake bilih fish (Mystacoleuseuspadangensis) is a fish originating from the Singkarak lake of West Sumatra Province. This study aims to obtain lactic acid bacterial candidate isolates from probiotic candidates from BilihDanauSingkarak fish which have not been utilized and have great potential to be developed. Each species of lactic acid bacteria has different probiotic effects, so selection and identification is needed to get a good probiotic strain. This research is a descriptive study and laboratory analysis to determine for sure the lactic acid bacteria found in bilih fish. LAB was isolated selectively using de Man Rogosa Sharpe Agar media. Selection was carried out by morphological observations and gram staining. Furthermore, testing the biochemical properties and identification of lactic acid bacteria in this study using the molecular method lderuiflcarlon with the 16s rRNA marker gene. The results of the isolation of lactic acid bacteria obtained the best IB1 isolates which were Gram-positive, Bacilli, catalase negative, homofermentative bacteria with the results of screening probiotic candidates having inhibitory power against pathogenic bacteria Escherichia coli O157 27.29 mm, resistance to bile salts, viability of lactic acid bacteria 77%, and The pH of the stomach, the viability of lactic acid bacteria was 30.56%, and the type of Lactobasillus fermentum was obtained. with a length of 1492 bp base.

Keywords: Lactic Acid Bacteria, Bilih Fish (*Mystacoleucuspadangensis*), Lake Singkarak, *Lactobacillus Fermentum*

I. Introduction

Lactic Acid Bacteria (LAB) have recently become a part of the subject matter in science, health, animal husbandry, agriculture and the food industry. LAB has been widely used in the fermentation of various types of food products both from animals, fish and plants which function as preservatives and have a positive impact on health and beauty. LAB has metabolite compounds which are very useful in maintaining the resistance of a product without reducing the quality of the initial product. According to (Mountzouris et al. 2010) LAB is a type of microorganism that is

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Received 05 March 2021; Accepted 01 April 2021.

included in the safe category (food grade microorganism) which is good for health (GRAS / Generally Recognized As Safe).

LAB also has functional properties that are beneficial to human health, namely as a probiotic. Probiotics are live microorganisms which, when consumed in sufficient quantities, can provide health benefits to their host (FAO 2006). Furri, (2008) explains that probiotics are supplements from live microbes that can replace the composition and replace the metabolic activity of natural gut microbes and regulate the reactivity of the immune system which is beneficial to health.

LAB potential needs to be isolated and screened for LAB, morphological identification, biochemical characterization, molecular DNA identification and purification so that it can be used as a probiotic candidate to maintain total health(Yunenshi, Syukur, and Purwati 2011). LAB potential that has been characterized and identified conventionally and molecularly and has been patented has a high value for application in various fields of science.

The presence of several LAB strains has been shown to have probiotic effects in humans. LAB can be isolated from animal and plant products such as animal products, namely milk, fish and others. One of them is Bilih fish (*Mystacoleucuspadangensis*), Lake Singkarak, Bilih fish is a potential source of animal protein because it has nutritional content in meat, nutritional content in Bilih fish meat is 13.02% protein, magnesium content in fresh bilih fish meat 0 , 18%, the phosphorus content of fresh bilih fish is 1.2%, fresh bilih fish meat contains 75.62% water content, the ash content of fresh bilih fish is 6.4%, bilih fish also has calcium content(Permata and Murtius 2015). Given the large nutritional content in bilih fish meat for human health, bilih fish is the right choice to be developed as a producer of probiotics. Through preliminary research conducted by the author, the Bilih fish (*Mystacoleucuspadangensis*) Lake Singkarak contains LAB, should be able to become one of the foodstuffs that can increase human immunity, especially during the new normal Covid-19 pandemic.

Clemente et al. (2012) stated that the main requirement for strains that can be used as probiotic agents is to have resistance to acids and bile so that they can reach the intestines and have the ability to stick to the intestinal mucosa. According to Syukur et al. (2013),probiotic bacteria have many benefits for human health, including in the immune system, intestinal system, urogenital system, reducing energy effects, and other benefits. Isolation and identification of LAB from Bilih fish (*Mystacoleucuspadangensis*) from Lake Singkarak, needs to be done to find out and obtain the LAB species found in Singkarak's lake bilih fish (*Mystacoleucuspadangensis*). Bilih fish (*Mystacoleucuspadangensis*) Lake Singkarak has potential as a probiotic which is useful for food, pharmacy and medicine. Based on this, it is very important to conduct research on "Isolation, Characterization and Molecular Identification of Lactic Acid Bacteria Isolated from Bilih Fish (*MystacoleucusPadangensis*) Lake Singkarak which has potential as a probiotic".

II. Materials And Methods

2.1. Research Material

a. Research Tools

The tools used during this research are autoclave, oven, hot plate, bunsen, petri dishes, test tubes, test tube racks, volumetric flasks, eppendorf tubes, erlenmeyer, grease paper, aluminum plates, incubators, object glass, aluminum foil, vortices. , analytical scale, measuring cup, beaker glas, hockeystick, pH meter, desiccator, kjehdal flask, loop needle, slide, microscope, magnetic stirrer, Lamina Flow, quebeccolony counter, micro pipette tip, anaerobic jar, micro pipette, centrifuge, PCR (Bio-Rad my cycler ™ thermal cycler, USA), loading day, agarose molding,

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electrophoresis (Power Pac Basic ™, USA), incubator shaker (Rocker NB-104).

b. Research Materials

The materials used in the research are Bilih Fish (Mystacoleucuspadangenis) Lake Singkarak from several places in the Lake Singkarak area, and materials commonly used in chemical and microbiological analysis such as: 23.5 grams Plate Count Agar (PCA) / Biolife Italia, Pepton Water (Biolife Italia), de Mann Rogosa Sharpe (MRS) Broth (Merck), de mannRagosa Sharpe (MRS) Agar (Merck), blue spritus, selenium, H2SO4, aquades, 30% NaOH, methyl red indicator, benzene, hydrogen peroxide (H2O2), HCl, Nutrient Agar, sterile distilled water, alcohol, crystal violet, iodine, safranin, ethanol, RNAse, F and R primers, dNTP, Taq Polymerase, buffer, agarose gel, TBE (Tris Boric EDTA), TAE (Tris Acid EDTA), RedSafe, Promega Kit Protocol, listeria enrichment broth, test bacteria (Listeria monocytogenes, Escherichia coli O157 and Staphylococcus aureus ATCC 25923), lugol, agarose, master mix and DNA ladder (Bioscience).

2.2. Research methods

This research is a descriptive research and laboratory analysis, namely the molecular identification of lactic acid bacteria from Bilih Fish (Mystacoleucuspadangenis) Lake Singkarak to obtain LAB isolates that are potential candidates for probiotics and the samples used as research materials were 3 different isolates with the same sample, namely IB. 1, IB.2 and IB.3.

a. Macroscopic identification of Bilih fish (*Mystacoleucuspadangenis*) in Lake Singkarakbacterial culture

De Man-Rogosa-Sharpe (MRS) broth (Merck) enriched media and MRS Agar (Merck) media were prepared. As much as 1 g of Bilih fish (*Mystacoleucuspadangenis*) in Lake Singkarakwas dissolved in 9 ml of MRS Broth solution in a test tube and vortexed until homogeneous for the initial 10^{-1} dilution of Bilih fish (*Mystacoleucuspadangenis*) in Lake Singkarak. Then, the solution was placed into an anaerobic jar and then incubated for 24 hours at 37 °C. After that, 1 ml (1000 µL) of the first dilution was taken, added into a test tube with 9 mL of MRS broth solution and then vortexed until homogeneous for the 10^{-2} dilution. This procedure was repeated until the 10^{-9} dilution is attained. From the last dilution, 100 µL of the sample was taken and spread on the MRS agar media. The inoculum was placed into the anaerobic jar and incubated for 48 hours at 37 °C. After 48 hours, a single colony of the LAB was observed. The total colony forming units (CFUs) was calculated using the Quebec colony counter (endang purwatiet al. 2016).

b. Morphology identification of Bilih fish (*Mystacoleucuspadangenis*) in Lake Singkarak bacterial culture

A loopful of bacterial culture was taken and then placed into a microscopic glass slide. Crystal violet dye was added on to the slide and then left to react for 1 minute. After that, it was rinsed with distilled water and then air-dried. Iodine drops were then added and left to react for 1 minute. Next, the slide was rinsed with distilled water and air-dried before dipping in ethanol for 20 minutes followed by counterstaining with safranin for 30 seconds. The result was observed under the microscope at 400× magnification.

c. Biochemical properties

The assay was carried out by inoculating the LAB isolates into 5 mL of MRS Broth (Merck) contained in test tubes. Durham tubes were inserted in inverted positions and then incubated for 48 hours at 37 °C. Then, the presence or absence of bubbles in the Durham tubes were observed.

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d. Acid resistance assay

Using 9 ml of MRS media broth, 1 ml bacterial culture was inoculated and incubated at 37 °C for 24 hours with the pH adjusted to 4 using 5N HCl addition. Furthermore, the dilution was carried out using the spread method to the MRS media and incubated at 37 °C for 48 hours. The CFU number of the bacteria that survived were calculated. The number of the survived colonies was expressed as LAB viability. The higher the LAB viability, the higher will be the LAB resilience against acid.

e. Bile salt resistance assay

One ml of bacterial culture was inoculated into 9 ml of MRS broth medium and incubated at 37 °C for 5 hours with 0.5% bile salt. The mixture was then serially diluted to 10^{-6}, spread on MRS agar medium and incubated at 37 °C for 48 hours. The number of bacteria that grew was calculated. The number of colony forming units (CFUs) was expressed as LAB viability count. The higher the LAB viability the higher will be the LAB resilience against bile salt.

f. Antimicrobial assay

Antimicrobial activity assays were carried out using the disk diffusion method with *Escherichia coli* O157, *Listeria monocytogenes,* and *Staphylococcus aureus* ATCC 25923 bacteria as test bacteria. One mL of LAB culture was placed into sterile Eppendorf tubes and then centrifuged at 10000 rpm for 5 minutes. 0.4 g of nutrient media was prepared (using 20 g of nutrient agar in 1000 mL of distilled water). Then 0.2% enriched bacteria colony was added to the medium and left to culture. The test strains mentioned were assayed for inhibition. Then, 50 µL of LAB supernatant was inserted onto the disk. The tested antibiotics were ampicillin 40 µL, and kanamycin 30 µL for controls compared to the supernatant. The petri plates used were then incubated anaerobically at 37 °C. The antibacterial activity was expressed as the diameter of the clear zones of inhibition caused by the antibiotics controls and the LAB supernatant.

g. Identification of LAB using 16S rRNA

Microbial DNA extraction was conducted using the Promega USA KIT. As much as 1000 µL of the single colony from MRS broth containing the LAB isolate was put in a new Eppendorf tube and then centrifuged at 14,000 rpm for 2 minutes. Then the pellet was taken, added with 480 µL of 50mM EDTA and 120 µL of Lysozyme. The mixture was incubated in 37 ^{o} C water bath for 60 minutes and then centrifuged for 2 minutes at 14,000 rpm. Then the supernatant was removed, the pellet was taken, then 600 µL of nuclei lysis solution was added and then homogenized. After that, the mixture was incubated at 80 ^{o}C for 5 minutes. After that, 3 µL of RNase Solution was added, homogenized, and incubated in 37 °C water for 60 minutes. Next, 200 µL of Protein precipitation solution was added, vortexed, incubated in ice for 5 minutes, and then centrifuged for 3 minutes at 14,000 rpm. The supernatant was transferred to a new tube and the pellet is removed, added with 600 µL isopropanol and homogenized, and then centrifuged for 2 minutes at 14,000 rpm. Then, the pellet and the supernatant were removed, 600 µL of 70% ethanol was added and homogenized, and centrifuged for 2 minutes at 14,000 rpm. Lastly, the pellet was taken and aerated for 15 minutes, and then rehydrated with 50 µL of rehydration solution for 30 minutes at 65^{o}C.

h. Preparation of PCR primary (16S rRNA)

The R primer (16S-1492R, Tm 47^{o}C, 5'GTT TAC CTT GTT ACG ACTT-3) and F primer (16S- 27F, Tm 54.3 ^{o}C, 5'AGA GTT TGA TCC TGG CTC AG-3) were prepared at 10pM concentrate. The primer was taken at 90 µL dH2O + 10µL of R and F primers. The R and F

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primers were dissolved in TE buffer with 100µM concentrate.

i. Preparation for 16S rRNA gene amplification

Preparation for 16S rRNA gene amplification are based on the primers for 16S rRNA Table 1 and the PCR programme Table 2.

Table 1. The primer 16S rRNA.

Composition	Volume (µl)
Master Mix	12.5 µl
Primer F	1 µl
Primer R	1 µl
DNA (Template)	1 µl
dH_2O	9,5 µl
Total	**25**

Note: All mixtures in the table are entered in a new eppendorf, the amount of H2OAdjusted to a total solution of 25 µl, mixed (flicked) and done by Shortspin (centifuge)

Table 2. The PCR programme.

Stages	Temperature	Time
Pre denaturasi	95 ^{0}C	2 minutes
Denaturasi	95 ^{0}C	45 seconds
Anneling	56 ^{0}C	45 seconds
Extention	72 ^{0}C	1 minutes 40 seconds
Final extention	72 ^{0}C	10 minutes
Cooling	4 ^{0}C	~

j. Preparation of 1.5% agarose for PCR electrophoresis

As much as 0.6 gram of agarose (obtained from 1.5% x 40 mL = 0.6 gram) was dissolved in 40ml of TAE buffer and then heated in a microwave for 30 seconds. After the solution was made lukewarm, 2 ul of redsafe / Gelview was added as a coloring agent. The agar height was about 0.5 mm and the electrophoresis comb arranged in the agar. The comb was removed after the agar was set.

k. Running gel electrophoresis

The agar was placed in the electrophoresis apparatus and then added with TAE solution until the agar is immersed. As much as 5µL sample and 5µL DNA ladder were injected into the agar well. The agarose run was set to 100V for 40 minutes and the result was seen under the UV light. After the PCR results were read, the amplified DNA was then purified to be sent to Genetika Lab for sequencing.

l. Analysis of sequencing data

The sequencing data analysis is performed using the DNASTAR software program. For the sequence alignment analysis, the obtained sequences were compared with those already deposited in the GeneBank (http://www.ncbi.nlm.nih.gov) using BLAST (Basic Local Alignment Search Tool). The phylogenetic analysis was done using MEGA v7.0 tools with the Maximum Likelihood and the kimura -2 mode algorithm.

III. Results and Discussion

3.1. Total colonies of Bilih Fish Lactic Acid Bacteria (Mystacoleucus

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padangensis) in Singakarak Lake

Colony calculations of lactic acid bacteria were carried out using a Quebec colony counter in order to determine the number of LAB colonies found in Bilih Fish (*Mystacoleucuspadangensis*) Lake Singakarak. The total number of LAB colonies can be seen in Table 3.

Table 3. Total Colonies of Bilih Fish Lactic Acid Bacteria *(Mystacoleucus padangensis*) Lake Singakarak

Sample	Total Lactic Acid Bacteria (x 10^6 CFU/g)
IB 1	64
IB 2	27
IB 3	44

The total calculation of lactic acid bacteria (LAB) aims to determine the number of lactic acid bacteria colonies found in Bilih Fish (*Mystacoleucuspadangensis*) Lake Singakarak. The total LAB colonies of Bilih Fish (*Mystacoleucuspadangensis*) in Lake Singkarak are shown in Figure 1.

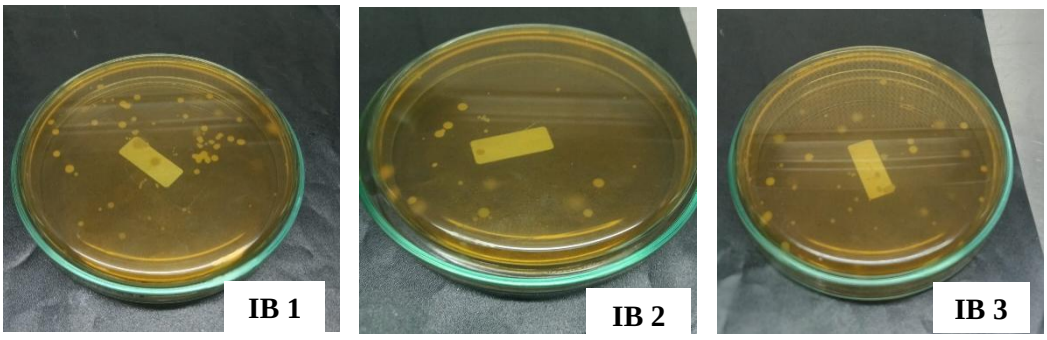

Figure 1. Bilih Fish BAL Colony on MRS Agar (IB1) sample from GuguakMalalo village, (IB 2) sample from Ombilin village Simawang, (IB 3) sample from Saniangbaka village

Based on the results of the research in Table 3 that the number of LAB colonies of Bilih fish (*Mystacoleucuspadangensis*) in Lake Singkarak can be calculated at dilution 106, we can see that the highest total colony of lactic acid bacteria was obtained from isolate IB 1 as much as 64 x106 CFU / g and the most a little on IB 2 isolates which only obtained a total LAB colony as much as 27x106 CFU / g.

The number of LAB colonies of Bilih fish (*Mystacoleucuspadangensis*) in Lake Singakarak is in accordance with the FAO / WHO (2002) criteria because LAB probiotic food produced is in the amount of 106 - 108 CFU / g. Yellowish white is the color of LAB obtained on MRS Agar from LAB isolates. These results are consistent with the research conducted by (Purwati, Syukur, and Hidayat 2005)which produced yellowish white LAB colonies on MRS Agar.

3.2 Morphological Identification of LAB Isolates

The identification of this study was carried out both macroscopically and microscopically, the results of microscopic and microscopic observations of this study can be seen in Table 4 below.

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Table 4. Morphological characteristics of LAB isolates of Bilih fish (*Mystacoleucus padangenis*)Lake Singakarak

Isolate	Macroscopic Identification					
	Color	Form	Size		Edge	Surface
IB 1	White-cream	Round	2,5	mm	Flat, smooth	Licin, cembung
IB 2	White-cream	Round	2,0	mm	Flat, smooth	Licin, cembung
IB 3	White-cream	Round	2,8	mm	Flat, smooth	Licin, cembung

Based on Table 4, it can be seen that the macroscopic observations (shape, size and color) of the Bilih fish isolate (*Mystacoleucuspadangensis*) in Singakarak Lake where all colonies are white - cream, round in shape with smooth and convex edges on MRS agar media. These results are consistent with the research conducted by (Purwati, Syukur, and Hidayat 2005)which states that LAB isolation colonies will produce yellowish white colonies on MRS Agar media. Furthermore, after carrying out macroscopic observations, it is followed by microscopic observations regarding the morphology of bacteria.The results of microscopic identification of lactic acid bacteria (LAB) with the Gram staining method showed that the LAB isolates isolated from Bilih fish (*Mystacoleucuspadangensis*) Lake Singakarak namely IB 1, IB 2 and IB 3 were Gram positive bacteria with rods (bacillus) (Figure 2). The same thing was also conveyed by Octarya, Syukur, and Purwati (2013)that crystal violet is alkaline so that it can bind to acidic microorganism cells. When washed with ethanol, Gram positive bacteria will still bind to the crystal violet-iodine complex, so that they are purple in color. The results of Gram staining of the LAB isolate of Bilih fish (*Mystacoleucuspadangensis*) Lake Singakarak from the research conducted can be seen in the Figure below.

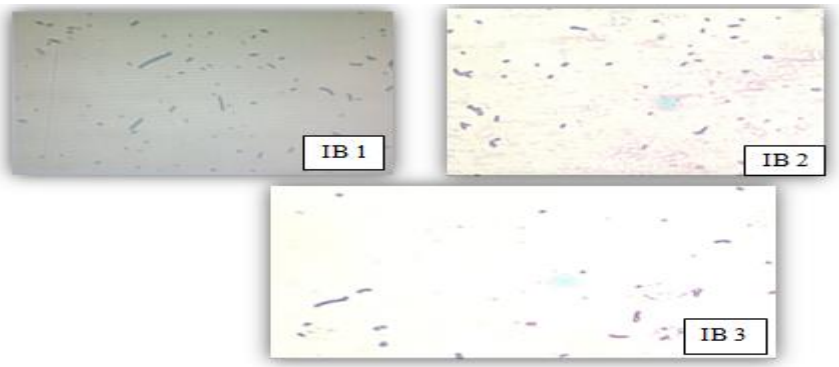

Figure 2. Gram staining of Lake Bilih fish isolates (*Mystacoleucuspadangensis*) Singakarak, (IB1) samples of bilih fish from GuguakMalalovillage, (IB 2) samplesFish Bilihfrom OmbilinSimawangvillage, (IB 3) sample of Bilih fish from Saniangbakavillage. Using a 10x strength ocular lens microscope and40x magnification objective lens

The results of Gram staining obtained for each isolate were Gram positive (+) bacteria. Observation under a microscope with a magnification of 40 times found the bacteria in the shape of a rod (bacil) and absorbing the violet color from the crystal violet.

3.3 Biochemical Test

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3.3.1. Catalase Test

The results of the catalase test for the LAB isolate of Bilih Fish (*Mystacoleucuspadangensis*) in Lake Singakarak can be seen in Table 5 below.

Table 5. Catalase test for LAB isolates of Bilih Fish (*Mystacoleucuspadangensis*) in Lake Singakarak

No.	Isolate	Catalase
1.	IB 1	Negative
2.	IB 2	Negative
3.	IB 3	Negative

Based on Table 5, all isolates in this study obtained negative results (-), observations were made by looking at the presence or absence of gas bubbles in the reviews of isolates that had been dripped with hydrogen peroxide (H2O2). The catalase test is used to determine the presence of the enzyme catalase in bacteria, where this enzyme plays a role in breaking down hydrogen peroxide (H2O2) into water and oxygen. The results of this study are the same as Delvia, Fridayanti, and Ibrahim (2015) who also found negative catalase in LAB isolates from mango fruit. Delvia, Fridayanti, and Ibrahim (2015)also found negative catalase in lactic acid bacteria isolated from scars.

3.3.2. Fermentation Type Test

The purpose of this test is to classify LAB into the homofermentative group or heterofermentative group. The results of the LAB isolate fermentation test are as shown in Table 6.

Table 6.Test of the type of fermentation of LAB isolates of Bilih Fish (*Mystacoleucuspadangensis*) Lake Singakarak

No.	Isolate	Homofermentatif	Heterofermentatif
1.	IB 1	+	-
2.	IB 2	+	-
3.	IB 3	+	-

The results obtained were that all LAB isolates from Bilih Fish (*Mystacoleucuspadangensis*) Lake Singakarak were homofermentative types, namely bacteria whose main product is lactic acid. The characteristic that can be seen in the inverted Durham tube on the test tube containing the isolate and MRS (Mann Ragosa Sharp) Broth is the absence of gas bubbles.

The results of this study are also in line with the opinion of Syukur dan Purwati (2013) which states that homofermentative LAB involves the Embden Meyernof-Parnas pathway, namely glycolysis which produces lactic acid, 2 moles of ATP from 1 glucose / hexose molecule under normal conditions, does not produce CO and produces biomass. twice as many cells as heterofermentative BAL.

3.4 LAB Screening for Bilih Fish (*Mystacoleucuspadangensis*) Lake Singakarak with Potential Probiotics

3.4.1 Antimicrobial Activity

Antimicrobial activity was carried out to determine whether LAB was able to inhibit the growth of pathogenic bacteria. The pathogenic bacteria tested in this study were *Escherichia coli O157, Staphylococcus aureus, and Listeria monocytogenes.* The diameter of the clear zone

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formed in each LAB isolate can be seen in Table 7 below:

Table 7. Diameter of Clear Zone for Antimicrobial Activity Test (mm)

No.	LAB isolates	Clear Zone Diameter (mm)		
		Escherichia coli O157	*S. aureus*	*L.monocytogenes*
1	IB 1	27.29	14.17	11.14
2	IB 2	15.16	12.09	9.16
3	IB 3	16.24	10.18	10.04

LAB isolate which had the largest inhibition zone in *Escherichia coli O157* was IB1 isolate with a diameter of 27.29 mm, while the lowest was IB2, namely 15.16 mm. The largest diameter of the zone of inhibition against *Staphylococcus aureus* bacteria in isolate IB1 was 14.17 mm, while the lowest was IB3, namely 10.18 mm. The largest diameter of the inhibition zone against *Listeria monocytogenes* bacteria which had the largest inhibition zone was isolate IB1 with a diameter of 11.14 mm, while the lowest was isolate IB2 of 9.16 mm. The clear zone of LAB isolates against pathogenic bacteria can be seen in Figure 10 below

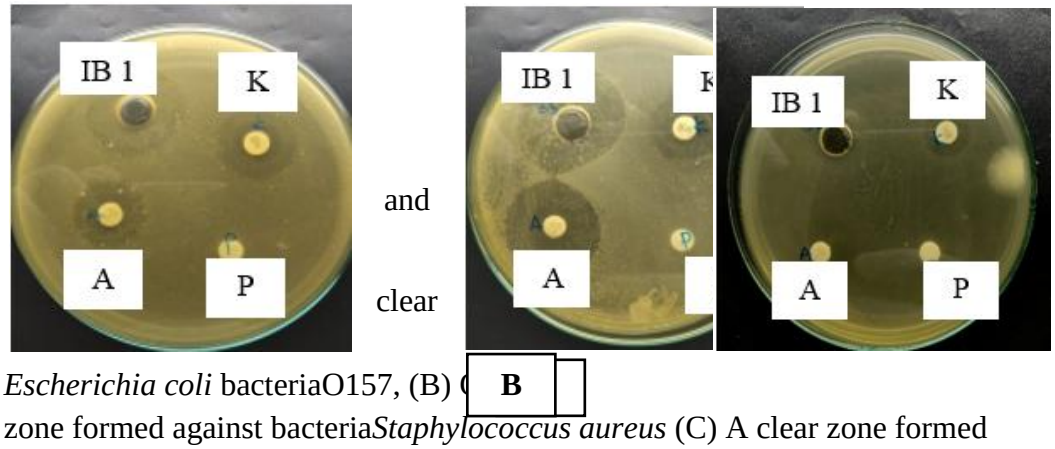

Figure 3. LAB antimicrobial activity test antibiotic test

Note: (A) A clear zone formed against *Escherichia coli* bacteriaO157, (B) A clear zone formed against bacteria*Staphylococcus aureus* (C) A clear zone formed against *Listeria monocytogenes* bacteria

The LAB isolate sample code IB1 had the largest inhibition zone, which was 27.29 mm in diameter against Escherichia coli O157, 14.17 mm in diameter for *Staphylococcus aureus* bacteria and 11.14 mm in diameter against *Listeria monocytogenes* bacteria. So that the IB1 isolate was continued with inhibition zone antimicrobial testing against pathogenic bacteria using penicillin, kanamycin, and ampicillin as a positive control.

The results of the antimicrobial activity test and antibiotic test for LAB isolates from Bilih Fish (*Mystacoleucuspadangensis*) Lake Singkarak IB1 can be seen in Table 8 below.

Table 8. Antimicrobial activity of LAB IB1 and antibiotic assays

Sample Code	Test Bacteria (mm)		
	Escherichia coli O157	*Staphylococcus aureus*	*Listeria monocytogenes*
IB1	27.29	14.17	11.14
Penicillin	-	-	-
Ampicillin	22.28	29.35	11.14

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Kanamycin	21.26	22.29	14,19

Based on the research results in Figure 3 and Table 8, it shows that the LAB IB1 isolate from Bilih Fish (*Mystacoleucuspadangensis*) Lake Singakarak was able to inhibit the three pathogenic test bacteria. The *Listeria monocytogenes* is 11.14 mm higher in diameter than the results of the study by Purwati et al. (2016) regarding the antimicrobial activity of LAB from curd which also used the *Listeria monocytogenes* test bacteria where the results of measuring the clear zone were 9 mm. In *Escherichia coli O157*, which is 27.29 mm in diameter, higher than the research by Saputri, Rossi, and Pato (2017) regarding the antimicrobial activity of LAB from soybean husks with *Eschericia coli O157* test bacteria, where the LAB inhibition zone against *Eschericia coli O157* is 8.31. mm. In S*taphylococcus aureus*, it is 14.17 mm in diameter. The results of this study were higher than those of Aritonang et al. (2017)regarding the antimicrobial activity of LAB Okara isolates against the *Staphylococcus aureus* test bacteria obtained a bacterial inhibitory power of 9.10 mm.

In the opinion of Morales et al. (2003) which states that the activity of the inhibition zone is grouped into four categories, namely: weak activity (<5mm), moderate (5–10mm), strong (> 10–20mm), very strong (> 20–30mm). Thereforeit can be said that the LAB IB 1 isolate from Bilih Fish (*Mystacoleucuspadangensis*) Lake Singakarak which has a clear zone is in the strong category against the three tested bacteria.

3.4.2. Resistance of LAB Isolate of Bilih Fish (*Mystacoleucuspadangensis*) Lake Singakarak Against Acidic pH

The acid resistance test was carried out successfully by indicating the presence or absence of colonies growing on the growing medium, with a dilution of up to 10-6. The results of the LAB isolate test results from Bilih Fish (*Mystacoleucuspadangensis*) Lake Singkarak to acid are presented in Table 9.

Table 9. LAB Isolate Resistance of Bilih Fish (*Mystacoleucuspadangensis*) in Lake Disclose Against Acidic pH

LAB Isolate Samples of Bilih Fish	Number of Bacterial Cells (CFU/ml)		Viability of LAB (%)
	Control	pH 3	
IB 1	84×10^6	26×10^6	30.56
IB 2	52×10^6	13×10^6	25.22
IB 3	102×10^6	10×10^6	10.58

Based on Table 13 shows that the test for acid resistance was carried out on LAB isolates from Bilih Fish (*Mystacoleucuspadangensis*) Lake Singkarak with control pH and pH 3 treatments. (without setting the pH 3) is 84 x 106 CFU / ml after being given a pH adjustment of 3 with the number of live bacterial cells, namely 26 x 106 CFU / ml, the highest LAB viability was 30.56%. Whereas for the lowest result was LAB isolates with sample code IB 3 with the number of live bacterial cells control (without pH 3 setting), namely 102 x 106 CFU / ml, after setting the pH of 3 live bacterial cells 10 x 106 CFU / ml, it resulted in viability. LAB for the lowest 10.58%.

The information obtained from the results of the acid pH resistance test showed that LAB isolates with sample code IB 1 were isolated from Bilih Fish (*Mystacoleucuspadangensis*) Lake

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Singkarak from GuguakMalalovillage as probiotic candidates because they were able to grow in the range of 26 x 106 acidic pH. This is in accordance with the opinion of Aritonang et al. (2017) stated that probiotic bacteria will have an effect on the intestinal environment if the total population of these bacteria reaches a minimum of 106-108 CFU / ml.

3.4.3. LAB Isolate Resistance of Singkarak Fish Bilih Fish (*Mystacoleucus padangensis*) Against Bile Salt Resistance

The resistance of the LAB isolate of Bilih Fish (*Mystacoleucuspadangensis*) Lake Singakarak to bile salts is an important requirement for probiotics as well as resistance to acids. The results of the test results of the LAB isolates of Bilih Fish (*Mystacoleucuspadangensis*) Lake Singkarak against bile salts are presented in Table 10.

Table 10. LAB Isolate Resistance of Bilih Fish (*Mystacoleucuspadangensis*) in Lake Singakarak Against Bile Salt

LAB Isolate Samples of Bilih Fish	Number of Bacterial Cells (CFU/ml)		Viability of LAB (%)
	Control	Oxgall 0.3%	
IB 1	116×10^6	95×10^6	77
IB 2	76×10^6	40×10^6	62
IB 3	101×10^6	62×10^6	58

Table 10 shows the number of bacteria living on the media with the addition of oxgall 0.3%. The most superior ability of LAB isolates from Bilih Fish (*Mystacoleucuspadangensis*) in Singakarak Lake was the sample code IB1 with the number of live bacterial cells control (without the addition of oxgall 0.3%), namely 116 x 106 CFU / ml. After being given the addition of oxgall 0.3% with the number of live bacterial cells, namely 95 x 106CFU / ml, so that the highest LAB viability was obtained, namely 77%. 3%) i.e. 101 x 106 CFU / ml. After being given the addition of oxgall 0.3% of live bacterial cells, namely 62 x 106 CFU / ml, resulting in the lowest LAB viability, namely 58%. LAB viability is the ratio of the number of microbes that grow before and after being given the adjustment of 0.3% oxgall addition which is expressed in the form of percent (%). This is in accordance with the opinion of Bezkorovainy (2001) which states that the resistance of lactic acid bacteria to bile salts is related to the bile salt hydrolase (BSH) enzyme which helps hydrolyze or break the C-24 N-acyl amide bonds that are formed between bile acids and amino acids in salt. conjugated bile.

Based on the results of the test for resistance to bile salts, it showed that the LAB isolate with sample code IB1 isolated from Bilih Fish (Mystacoleucuspadangensis) from GuguakMalalo village was the most excellent candidate for probiotics.

3.5 Identification of Lactic Acid Bacteria Isolates from Bilih Fish (*Mystacoleucuspadangensis*) Lake Singkarak with 16S rRNA

3.5.1 Results Amplification of the 16S rRNA gene by PCR

Resultsof this electrophoresis indicate that the PCR activities that have been carried out have succeeded in amplifying the 16S rRNA gene area of LAB isolates from Bilih Fish (Mystacoleucuspadangensis) Lake Singkarak. This can be seen with the appearance of PCR product fragments with a size of 1429 bp which is the expected size if you use a combination of 27F AGAGTTTGATCCTGGCTGAG primers for the forward direction with 1492 R GTTTACCTTACGACTT primers for the reverse direction. Furthermore, sequencing using a

Annals of R.S.C.B., ISSN:1583-6258, Vol. 25, Issue 4, 2021, Pages. 8581 - 8596
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reverse primer, the sequencing results were obtained as many as 885 nucleotides.The results of electrophoresis of LAB isolate PCR products obtained are in Figure 11 below:

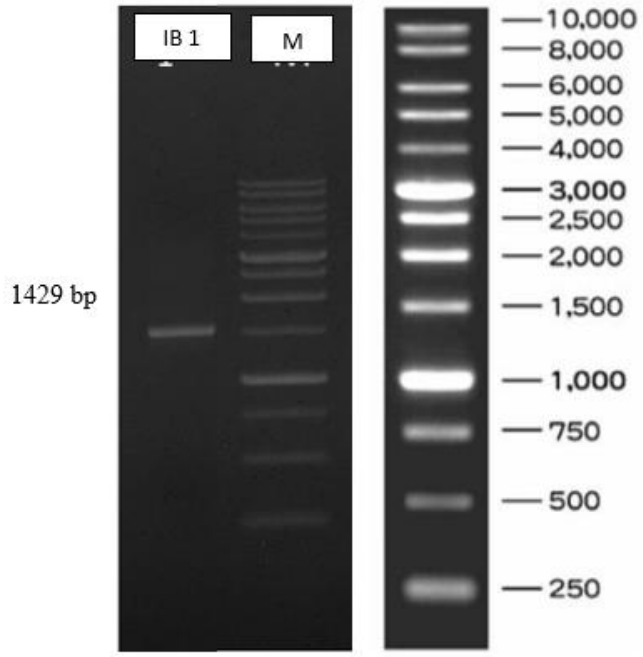

Figure 4. The results of PCR electrophoresis of LAB isolates from Bilih Fish (*Mystacoleuspadangensis*) Lake Singkarak (M = Marker, IB1 = LAB Isolate Bilih fish (*Mystacoleucuspadangensis*) Lake Singkarak

The results obtained in this study (Figure 4) show the results of 1429 bp DNA amplification on agarose gel. This indicates that the specific primers used in this study were able to identify bacteria down to their strain level.

3.5.2. Analysis of 16S rRNA Gene Sequence Isolate from Bilih Fish (*Mystacoleucuspadangensis*) in Singkarak Lake

The results of sequencing of IB1 isolates were compared with Gene Bank data using the Basic Local Allignment Search Tool (BLAST) program to determine the homology of sequences carried out online on the NCBI website (http://www.ncbi.nlm.nih.gov).

Sequence Assembly

1TTGATTGATGGTGCTTGCACCTGATTGATTTTGGTCGCCAACGAGTGGCGGACGGGTGAG

61TAACACGTAGGTAACCTGCCCAGAAGCGGGGGACAACATTTGGAAACAGATGCTAATACC

121GCATAACAGCGTTGTTCGCATGAACAACGCTTAAAAGATGGCTTCTCGCTATCACTTCTG

181GATGGACCTGCGGTGCATTAGCTTGTTGGTGGGGTAATGGCCTACCAAGGCGATGATGCA

241TAGCCGAGTTGAGAGACTGATCGGCCACAATGGGACTGAGACACGGCCCATACTCCTACG

301GGAGGCAGCAGTAGGGAATCTTCCACAATGGGCGCAAGCCTGATGGAGCAACACCGCGTG

361AGTGAAGAAGGGTTTCGGCTCGTAAAGCTCTGTTGTTAAAGAAGAACACGTATGAGAGTA

421ACTGTTCATACGTTGACGGTATTTAACCAGAAAGTCACGGCTAACTACGTGCCAGCAGCC

481GCGGTAATACGTAGGTGGCAAGCGTTATCCGGATTTATTGGGCGTAAAGAGAGTGCAGGC

541 GGTTTTCTAAGTCTGATGTGAAAGCCTTCGGCTTAACCGGAGAAGTGCATCGGAAACTGG

601ATAACTTGAGTGCAGAAGAGGGTAGTGGAACTCCATGTGTAGCGGTGGAATGCGTAGATA

661TATGGAAGAACACCAGTGGCGAAGGCGGCTACCTGGTCTGCAACTGACGCTGAGACTCGA

Annals of R.S.C.B., ISSN:1583-6258, Vol. 25, Issue 4, 2021, Pages. 8581 - 8596
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721AAGCATGGGTAGCGAACAGGATTAGATACCCTGGTAGTCCATGCCGTAAACGATGAGTGC

781TAGGTGTTGGAGGGTTTCCGCCCTTCAGTGCCGGAGCTAACGCATTAAGCACTCCGCCTG

841GGGAGTACGACCGCAAGGTTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGG

901AGCATGTGGTTTAATTCGAAGCTACGCGAAGAACCTTACCAGGTCTTGACATCTTGCGCC

961AACCCTAGAGATAGGGCGTTTCCTTCGGGAACGCAATGACAGGTGGTGCATGGTCGTCGT

1021CAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTTACTAGT

1081TGCCAGCATTAAGTTGGGCACTCTAGTGAGACTGCCGGTGACAAACCGGAGGAAGGTGGG

1141GACGACGTCAGATCATCATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGGACGG

1201TACAACGAGTCGCGAACTCGCGAGGGCAAGCAAATCTCTTAAAACCGTTCTCAGTTCGGA

1261CTGCAGGCTGCAACTCGCCTGCACGAAGTCGGAATCGCTAGTAATCGCGGATCAGCATGC

1321CGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGAGAGTTTGTAA

1381CACCCAAAGTCGGTGGGGTAACCTTTAGGAGCCAGCCGCCTAAGGGGAC

Figure 5.The results of nucleotide sequencing of LAB isolates from Bilih Fish (*Mystacoleucuspadangensis*) Lake Singkarak (IB1)

Based on the results of analysis using BLAST carried out at (http://www.ncbi.nlm.nih.gov), the type of bacteria IB 1 isolate from Bilih Fish (Mystacoleucuspadangensis) Lake Singkarak has 99% similarities with Lactobacillus fermentum strains 4 - 17 16S. This indicates that the type of LAB isolate bacteria in Bilih Fish (Mystacoleucuspadangensis) Lake Singkarak which was found to be close to Lactobacillus fermentum strains 4-17 16S with Percent Identy 99.93, according to the research of Hagström, Pinhassi, and Zweifel (2000). isolates with more than 97% similarity of the 16S rRNA sequence could represent the same species. The results of the BLAST analysis of the study showed that the sequence using the 16S rRNA gene for IB1 isolates can be seen in Table 11 below.

Table 11. BLAST results of LAB isolates of Bilih Fish (*Mystacoleucus padangensis*)Lake Singkarak (IB1)

No	Description	Query Cover (%)	Acession Number	Percent Ident (%)
1	*Lactobacillus fermentum* strain: 6052 16S	99	MT463632.1	99.93
2	*Lactobacillusfermentum* strain:5165 16S	99	MT510256.1	99.93
3	*Lactobacillus fermentum* strain: 5194 16S	99	MT463446.1	99.93
4	*Lactobacillusfermentum* strain: 4887 16S	100	MT505630.1	99.86
5	*Lactobacillus fermentum* strain: 4-17 16S	99	KY435725.1	99.93
6	*Lactobacillus fermentum* strain: IMAU32708 16S	99	KF149376.1	99.93
7	*Lactobacillus fermentum* strain: 1692 16S	100	MT597564.1	99.86
8	*Lactobacillus fermentum* strain: 7251 16S	100	MT516047.1	99.86

Annals of R.S.C.B., ISSN:1583-6258, Vol. 25, Issue 4, 2021, Pages. 8581 - 8596
Received 05 March 2021; Accepted 01 April 2021.

No	Description	Query Cover (%)	Acession Number	Percent Ident (%)
9	*Lactobacillus fermentum* strain: 5154 16S	100	MT510246.1	99.86
10	*Lactobacillus fermentum* strain: 4820 16S	100	MT505590.1	99.86

Based on Table 11, it has 99% similarity to the genomic sequence and partial strains of lactobacillus. In accordance with Notredame (2007) statement that a sequence can be said to be homologous if it has similitude of more than 70%, the analysis carried out on BLAST is then visualized using the MEGA v7.0 application tools which have previously been aligned using the Bioedit application first.

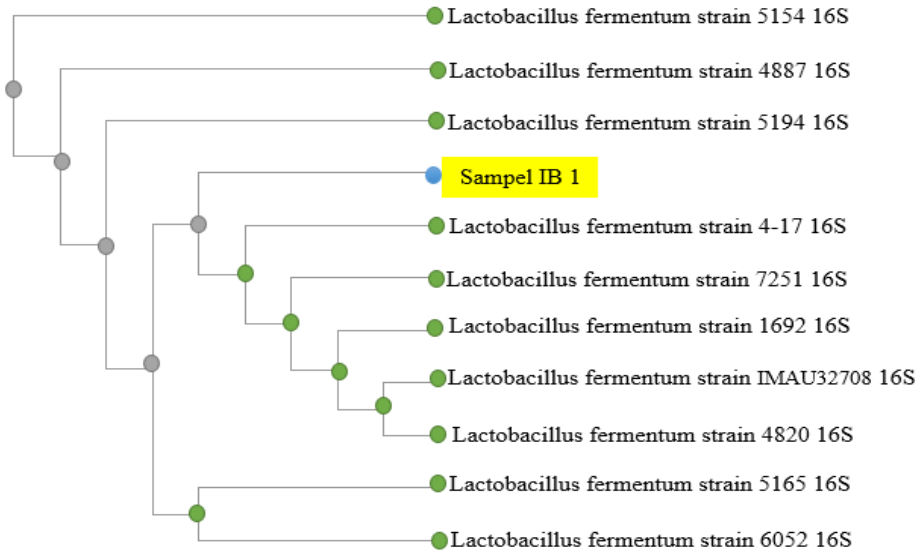

Figure 6.Pylogenetic tree of LAB isolates of Bilih Fish (*Mystacoleucus padangensis*) Lake Singkarak (IB1)

Based on the PCR (Polymerase Chain Reaction) results that have been carried out and after being analyzed using BLAST, the type of bacteria isolate IB 1 Bilih Fish (*Mystacoleucuspadangensis*) Lake Singkarak has 100% similarity with Lactobacillus fermentum strains 4-17 16S. In accordance with Notredame (2007) statement that a sequence can be said to be homologous if it has similarity of more than 70%, while the sequence equation between 93% - 97% can represent the identity of bacteria at the genus level but different species. The phylogenetic tree obtained shows the close relationship between Lactobacillus fermentum bacteria. A very clear pattern was seen in which the bacterial samples formed a monophyletic group with L. *fermentum strains* 4-17 16S, 7251 16S, 1692 16S, IMAU32708 16S and 4820 16S. This indicates the reliability of the very close kinship between these bacteria from the same ancestor.

Syukur et al. (2013)stated that the Lactobacillus group of bacteria is included in the group of "lactic acid bacteria" and is the most widely used as a probiotic agent because the end product of lactic acid metabolism comes from sugar fermentation and is an anaerobic bacteria which is

Annals of R.S.C.B., ISSN:1583-6258, Vol. 25, Issue 4, 2021, Pages. 8581 - 8596
Received 05 March 2021; Accepted 01 April 2021.

widely found in fermented foods such as yogurt, cheese, pickles, kimchi, and stock fish. Even though some strains are closely related, their ability to develop and grow in a certain environment depends on their natural habitat. It can be concluded that Lactobacillus fermentum can be used as a probiotic.

IV. Conclusion

Bilih fish (*Mystacoleucuspadangensis*) in Lake Singkarak, is one of the dominant fish living in Lake Singkarak which is rich in LAB. LAB isolated from Bilih Fish (*Mystacoleucuspadangensis*) Lake Singkarak has the potential to be a natural, non-pathogenic, viable probiotic in medium with low pH and high concentrated bile salts and has antibacterial activity. Molecular and bioinformatics analysis showed that LAB in Singkarak Lake Bilih Fish (*Mystacoleucuspadangensis*) is *L. fermentum* which has antioxidant properties and health benefits as well as additional therapy to overcome the side effects of antibiotics in the gastrointestinal tract.

ACKNOWLEDGMENT

This study was supported by the Laboratory of Livestock Products Technology, Faculty of Animal Husbandry, Andalas University, Padang. The author would like to thank the Institute for Community Service Research (LPPM) Andalas University through the excellent basic research scheme for professor publication research clusters with contract number T / 15 / UN.16.17 / PT.01.03 / KO-PDU-KRPIGB / 2020, Prof. Drh. Hj. EndangPurwati, MS, Ph.D.

Practical Application: Bilih Fish (*Mystacoleucuspadangensis*) Lake Singkarak is one of the dominant fish that live in Lake Singkarak, West Sumatra Province which is rich in LAB and has the potential as a probiotic which is useful for health, livestock, agriculture and the food industry.

REFERENCES

1. Aritonang, S N et al. 2017. “Isolation and Identification of Lactic Acid Bacteria from Okara and Evaluation of Their Potential as Candidate Probiotics.” *Pakistan J. Nutri* 16: 618–28.
2. Bezkorovainy, Anatoly. 2001. “Probiotics: Determinants of Survival and Growth in the Gut.” *The American journal of clinical nutrition* 73(2): 399s-405s.
3. Clemente, Jose C, Luke K Ursell, Laura Wegener Parfrey, and Rob Knight. 2012. “The Impact of the Gut Microbiota on Human Health: An Integrative View.” *Cell* 148(6): 1258–70.
4. Delvia, Fila, Aditya Fridayanti, and Arsyik Ibrahim. 2015. “Isolasi Dan Identifikasi Bakteri Asam Laktat (BAL) Dari Buah Mangga (Mangifera Indica L.).” In *Proceeding of Mulawarman Pharmaceuticals Conferences*, , 114–20.
5. Furri, M. 2008. “Differences in the Confluence of Mesial Canals in Mandibular Molar Teeth with Three or Four Root Canals.” *International endodontic journal* 41(9): 777–80.
6. Hagström, Åke, Jarone Pinhassi, and Ulla Li Zweifel. 2000. “Biogeographical Diversity among Marine Bacterioplankton.” *Aquatic Microbial Ecology* 21(3): 231–44.
7. Morales, Glauco et al. 2003. “Secondary Metabolites from Four Medicinal Plants from Northern Chile: Antimicrobial Activity and Biotoxicity against Artemia Salina.” *Journal of the Chilean Chemical Society* 48(2): 13–18.

Annals of R.S.C.B., ISSN:1583-6258, Vol. 25, Issue 4, 2021, Pages. 8581 - 8596
Received 05 March 2021; Accepted 01 April 2021.

8. Mountzouris, K C et al. 2010. “Effects of Probiotic Inclusion Levels in Broiler Nutrition on Growth Performance, Nutrient Digestibility, Plasma Immunoglobulins, and Cecal Microflora Composition.” *Poultry science* 89(1): 58–67.
9. Notredame, Cédric. 2007. “Recent Evolutions of Multiple Sequence Alignment Algorithms.” *PLoS Comput Biol* 3(8): e123.
10. Octarya, Zona, Sumaryati Syukur, and Endang Purwati. 2013. “Purifikasi Parsial Enzim Ekstraseluler (Anoxybacillus Sp.) Yang Diisolasi Dari Sumber Air Panas Bukit Kili Solok Serta Aplikasinya Untuk Menghidrolisis Limbah Berserat.” *Jurnal Natur Indonesia* 15(2): 106–14.
11. Permata, Deivy Andhika, and Wenny Surya Murtius. 2015. “Kandungan Zat Gizi Dan Bakteri Proteolitik Pada Produk Olahan Ikan Bilih.” *Jurnal Teknologi Pertanian Andalas* 19(1): 10–14.
12. Purwati, E et al. 2016. “Manfaat Probiotik Bakteri Asam Laktat Dadiah Menunjang Kesehatan Masyarakat.” *Penerbit Lembaga Literasi Dayak, Tangerang Banten*.
13. Purwati, E, S Syukur, and Z Hidayat. 2005. “Lactobacillus Sp. Isolasi Dari Biovicophitomega Sebagai Probiotik.” *Proceeding Lembaga Ilmu Pengetahuan Indonesia, Jakarta*: 24–25.
14. Rusmana, Iman, Antonius Suwanto, and Nisa Rachmania Mubarik. 2012. “Senyawa Antimikroba Yang Dihasilkan Oleh Bakteri Asam Laktat Asal Bekasam.” *Jurnal Akuatika* 3(2).
15. Saputri, Megawati, Evy Rossi, and Usman Pato. 2017. “Aktivitas Antimikroba Isolat Bakteri Asam Laktat Dari Kulit Ari Kacang Kedelai Terhadap Escherichia Coli Dan Staphylococcus Aureus.”
16. Syukur, Sumaryati, Benrward Bisping, Z Aneloi Noli, and Endang Purwati. 2013. “Antimicrobial Properties and Lactase Activities from Selected Probiotic Lactobacillus Brevis Associated with Green Cacao Fermentation in West Sumatra, Indonesia.” *J Prob Health* 1(4).
17. Urnemi, Urnemi et al. 2016. “Potensi Bakteri Asam Laktat Sebagai Kandidat Probiotik Antimikroba Patogen Asal Fermentasi Kakao Varietas Criollo.” *Jurnal Riset Teknologi Industri* 6(12): 67–76.
18. Yunenshi, Febria, S Syukur, and E Purwati. 2011. “Pengaruh Pemberian Probiotik Pediococcus Pentosaceus Asal Fermentasi Kakao Hibrid Terhadap Penurunan Kolesterol Telur Itik Pitalah.”