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Antimicrobial Potential of *Limosilactobacillus Fermentum* Isolated from Bilih Fish (*Mystacoleucus padangensis*) of Singkarak Lake, West Sumatera, Indonesia

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Abstract

Background and Objective: Lactic acid bacteria have recently become one of the major topics of discussion in fields of health, food industry, science, animal husbandry and agriculture. Lactic acid bacteria have been widely used in fermentation of various types of food products from animals, fish and plants that act as preservatives and include positive effects on human health and beauty. One source of lactic acid bacteria is *Mystacoleucus padangensis* fish from Singkarak Lake, West Sumatera, Indonesia, where a probiotic bacterium of *Limosilactobacillus fermentum* with antimicrobial potential is isolated. The aim of this study was to assess antimicrobial potential of *Limosilactobacillus fermentum* isolated from Bilih fish from Singkarak Lake, Indonesia.

Material and Methods: Methods of this study were as follows: isolation of lactic acid bacteria from Bilih fish from Singkarak Lake, followed by antimicrobial activity assessment of the raw bacteriocin supernatant. Then, 16S rRNA was used to assess species of the lactic acid bacteria isolates. From the three samples, one sample of Isolate IB1, with potential antimicrobial activity was reported.

Results and Conclusion: Results of the study showed that the morphological and biochemical characteristics of lactic acid bacteria included Gram-positive, bacilli form and catalase-negative belonged to the group of homofermentative bacteria. The greatest antimicrobial activities were shown by IB1 *Escherichia coli* 0157:H7 (27.29 mm), *Staphylococcus aureus* ATCC 25923 (14.17 mm) and *Listeria momocytogenes* CFSAN 004330 (11.44 mm) while the diameter of inhibition zone by the supernatant of Lactic acid bacteria *Escherichia coli* 0157:H7 crude bacteriocin was 16.89 mm while *Staphylococcus aureus* ATCC 25923 did not form inhibition zone. The observed antimicrobial activity was 17.19 mm for *Listeria monocytogenes* CFSAN 004330 at neutral pH. Results of the molecular identification using 16S rRNA showed that the isolated lactic acid bacteria included similarities with *Limucilactbacillus fermentum* strain 17059 16S, which included antimicrobial potentials against pathogenic bacteria. *Limosilactobacillus fermentum* 17059 16S can be used as an antidiarrheal and antityphoid agent in humans and as a natural preservative in foods.

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1. Introduction

A well-known source of probiotics is the group of lactic acid bacteria (LAB), which are Generally Recognized as Safe. The LAB can survive and form colonies in the intestine then produce lactic acid and other metabolites and stimulate the body immune response [1]. In fact, LAB is a group of bacteria capable of converting carbohydrates (glucose) into lactic acid. Moreover, LAB are able to produce hydrogen peroxide (H_2O_2) and bacteriocin, which include antagonistic characteristics against pathogenic bacteria. Technically, LAB can be isolated from natural and traditional fermented food products and fermented vegetables. Potential LAB as probiotics must first be identified, including identification of

bacterial morphology, biochemical characterization and molecular DNA. Then, LAB can be used as probiotic candidates, which are useful for protecting the body health from viruses and other microbes [2]. Potential LAB have been identified and characterized using molecular and conventional methods and are already used in various disciplines of animal husbandry, medicine and agriculture. Presence of several types of LAB strains is verified to include probiotic effects on humans, especially *Lactobacillus* Sp., which is part of the normal flora of the human digestive tract [2]. Lactobacilli are probiotic providing beneficial effects on health such as controlling diarrhea [3], stimulating the immune system [4], lowering cholesterol levels [5] and preventing colon and intestine cancers [6] and treatment of atopic dermatitis in children [7]. One example is in animal products of Bilih fish *Mystacoleucus* (*M.*) *padangensis* from Singkarak Lake, West Sumatera, Indonesia. Based on the results of several studies on Bilih fish, this fish is a source of animal proteins that include potentials of developing in form of foods or other processed products. Bilih fish includes nutritional contents of 13.02% of protein, 0.18% of magnesium, 1.2% of phosphorus, 75.62% of water and 6.4% of ash. Moreover, Bilih fish includes calcium [8]. This fish is a fermented fish with the addition of salt with levels that do not include definite standards, depending on the location of manufacture and storage time. Due to its large nutritional contents for human health, Bilih fish includes the potential of developing as a probiotic producer.

From previous studies by the current authors, Bilih fish of Singkarak Lake contains the potential for probiotics and can be used as a supplement for laying quail to improve production performance and decrease cholesterol levels. Foods that are low in cholesterol can increase human immunity, especially during the Covid-19 pandemic. Several studies have been conducted by researchers around the world regarding the content of LAB in fish. One example is bales isolated from fermented marsh finfish by adding virgin coconut oil which succeeded in finding that there were bales with antimicrobial activity [9]. These LAB included

Lactiplantibacillus (*L.*) *plantarum* T2565, B765, N2352, B1465, *Lactobacillus* (*L.*) *pentosus* B2555 and *Pediococcus* (*P.*) *pentosaceus* B1666. Furthermore, [10] it was detected that the antimicrobial activity of LAB isolates against the pathogenic bacteria of *Staphylococcus* (*S.*) *aureus* was caused by the organic acids produced by the LAB. Furthermore, lactic acid bacteria isolated from Bekasam which is a processed food from fish originating from Jambi Province has antimicrobial bacterial activity that has been successfully researched and patented which bacteria that have been found include *L. pentosus* BS15, *L. plantarum* 1 BS22 and 1 BL12. [11]. Other studies also tested the antibacterial activity of lactic acid bacteria isolated from Bekasam against *S. aureus* ATCC 25923, *Escherichia* (*E.*) *coli* ATCC 25922 and *Salmonella* (*S.*) *sp* [12]. However, previous studies did not use similar types of fish and hence could result in various microbial profiles when compared to this study. This study assessed potentials of LAB in Bilih fish from Singkarak Lake, West Sumatera, and Indonesia, which was an endemic fish species of the lake and included antimicrobial characteristics. Furthermore, the micro-organisms were potential sources of bacteriocins, which could be used as probiotics and biopreservatives.

2. Materials and Methods

2.1. Research Equipment

The tools used in this study include anaerobic jar, autoclave, Bunsen, Petri dish, agarose mold, Erlenmeyer electrophoresis (PowerPac Basic, USA), measuring cup, hockey stick, hot plate, incubator shaker (Rocker NB-104), incubator, loop needle, laminar air flow cabinet (Erlab Captair Biocap, Biocap, France), slide, microscope, micropipette, polymerase chain reaction (PCR) (Bio-Rad My Cycler, Bio-Rad, USA), centrifuge, TLC apparatus, spectrophotometer, test tube, test tube rack, microtube, vortex, analytical balance, Quebec colony counter and micropipette tip. Tools used in the analysis included analytical scales.

2.2. Research Materials

Materials used in this study included Bilih fish samples from several locations of Singkarak Lake area as well as materials commonly used in chemical and microbiological analyses such as sterile distilled water (DW), alcohol, benzene, blue spritus, buffer, MRS broth (Merck, Germany), MRS agar (Merck, Germany), 70% ethanol, Mueller-Hinton agar (MHA), pepton water, sprite, cristal violet, iodine, safranin, 1× tris ethylene diamine tetra acid (tris EDTA), lysozyme, RNAse, 10× buffer, agarose gel, 1× TBE tris-boric-EDTA, TAE (tris acid EDTA), ddH_2O, Promega master mix Kit, DNA ladder (Bioscience), selenium, H_2SO_4, 30% NaOH, methyl red indicator, H_2O_2, HCl, nutrient agar, crystal violet, iodine, safranin, ethanol, RNAse, primers F and R, dNTP, Taq polymerase, agarose gel, RedSafe, Listeria enrichment broth, lugol and test bacteria including *Listeria* (*L.*) *monocytogenes* CFSAN 004330, *E. coli* O157:H7 and *S. aureus* ATCC 25923.

2.3. Bilih fish of Singkarak Lake

Bilih fish is an endemic fish that lives in Singkarak Lake, which is processed into traditional foods as Bilih goreng with a simple manufacturing process. The ingredients consist of fish, rice and salt. Fish is cleaned, added with salt and rice and stored in containers with tightly closed cap. Containers were stored at room temperature for three days (Figure 1).

2.4. Sampling

Samples of Bilih fish were collected from the fishermen in Singkarak Lake with various fishing gear. Samples 1 and

2 were collected from fishermen whose fishing gear was the bagan (floating net); in which, the operation was carried out in deep waters in the morning. The charts owned by fishermen vary in size but were mostly 6 × 6 cm. Bilih fish were fishered at 5 am. The floating net includes a length of 200 m and a depth of 50 m. The fishing gear was installed at 4 am and lifted at 9 am. The fish were then separated based on the size of the mesh and were immediately transferred into a cool box and carried to the laboratory. There were several sampling locations for Bilih fish in Singkarak Lake with the criteria that the location was easily accessible. Sampling locations are shown in Table 1. Map of the Bilih fish sampling location in Singkarak Lake, Tanah Datar and Solok Regencies, is shown in Figure 2.

Table 1. Sampling of Bilih fish (*Mystacoleucus padangensis)* from Singkarak Lake, West Sumatera, Indonesia

Sample	Location	Fish type	Type of fishing gear
IB1	Nagari Guguak Malalo	Bilih Fish	Chart (floating net)
IB2	Nagari Simawang		
IB3	Nagari Singkarak		

a b c

Figure 1. a) Bilih fish harvested from Singkarak Lake; b) Bilih fish stored in a jar; c) one gram of salt was added to Bilih fish andstored in a closed container at room temperature for three days

Figure 2. Location map of Singkarak Lake, West Sumatera

2.5. Isolation and identification of lactic acid bacteria

2.5.1. Total lactic acid bacteria isolated from the Bilih fish

The necessary equipment such as Petri dishes, test tubes, Erlenmeyer flasks, microtubes, micropipette tips and hockey sticks were sterilized at 121 ºC for 15 min at 15 lbs using autoclave. Then, MRS broth (Merck, Germany) was prepared (52.2 g MRS broth powder in 1 l of DW), homogenized at 100 ºC using magnetic stirrer with hot plate and autoclaved (121 ºC, 15 min, 15 lbs) and prepared for making MRS agar media (Merck, Germany) (68.2 g MRS agar powder in 1000 ml of DW). This was homogenized at 100 ºC using magnetic stirrer with hotplate, autoclaved, cooled

down to ±55 ºC and poured into Petri dishes of ±8 ml. Then, 1 g of Bilih fish was separated using sterile spoon, dissolved in a test tube containing 9 ml of MRS broth and vortexed. This was called 10^{-1} dilution. Then, 100 µl of this dilution were transferred into a microtube containing 900 µl of MRS broth and vortexed. Dilution was then called 10^{-2} dilution. This continued until preparation of 10^{-7} dilution. Then, 100 µl of the sample were spread on a Petri dish containing MRS agar under a laminar air flow cabinet. The inoculum was stored in an anaerobic jar and incubated at 37 ºC for 48 h. After 48 h, LAB colonies were counted using Quebec Colony Counter. The calculation results of LAB colonies were multiplied by ten [13].

2.5.2. Isolation of lactic acid bacteria from fermented Bilih fish

Briefly, 1 ml fermented Bilih fish was diluted into 9 ml of MRS broth and vortexed. Then it was transferred into an anaerobic jar and incubated at 37 ºC for 24 h using incubator. Results of the 10^{-1} dilution were sampled as 1 ml samples, transferred into test tubes containing 9 ml of MRS broth and then vortexed. Result of this dilution was called the 10^{-2} dilution. Dilution was carried out until preparation of 10^{-7} dilution. A total of 100 µl of samples from the 10^{-7} dilution were spread on a petri dish containing MRS agar. The inoculum was stored using anaerobic container. Incubation was carried out at 37 °C for 48 h using incubator. A single LAB colony was transferred to MRS agar for colony purification and incubated at 37 ºC for 24 h [13].

2.6. Antimicrobial activity of the lactic acid bacteria

A modified method was used to assess antimicrobial activity of the LAB against pathogens [13]. The method included sterile essay paper diffusion method. In summary, 1 ml of the LAB cultivar was centrifuged at 2817.36 ×g for 5 min at 4 °C. Then cell-free supernatant was collected from the LAB, which were cultured in MRS broth at 37 °C for 24 h under anaerobic conditions, and centrifuged at 7826 ×g for 5 min at 4 °C. Supernatant was used to assess the microbial activity. Briefly, 20 µl of the pathogenic bacteria were mixed with 20 ml of liquid nutrient agar (Merck, Germany). Pathogenic bacteria of *E. coli* O157:H7, *S. aureus* and *L. monocytogenes* were cultured aerobically at 40 ºC. Mixture was homogenized, poured into sterile Petri dishes and set for 30 min. Then, 20 µl of the LAB supernatant was dropped onto sterile test paper as positive control (including penicillin 10 g, kanamycin 30 g and ampicillin 10 g) and incubated at 37 °C for 24 h anaerobically. Inhibition (clear) zones around the test paper were reported after incubation.

2.7. Antimicrobial assay of bacteriocin in the supernatant

Briefly, 1 ml of the culture was incubated in 9 ml of MRS solution at 37 ºC for 24 h. Then, bacteria for mix with MRS solution were centrifuged at 15338.96 ×g for 5 min. Then, filtering step for the supernatant was carried out through 0.22-µl membrane filters. Cell-free supernatant was adjusted to pH 6.5 with 1 N NaOH solution to eliminate the inhibitory effect of organic acids [14]. The pathogenic bacteria were aerobically incubated at 37 °C for 1 days. Then, 0.2% of the pathogenic bacteria culture was added to 20 ml of MHA solution at 50 °C. After this was solidified, 6-mm wells were made in the media using cork drill. Furthermore, 50 µl of the supernatant were transferred into each well and allowed to set for 15–10 min. Incubation was carried out at 37 °C for 1 days under aerobic conditions. A verification assay of the antimicrobial bacteriocin in the supernatants was carried out at 100 ºC with several time intervals of 15, 30 and 60 min. After being heated, the LAB supernatant was assessed for its ability to inhibit indicator bacteria of *E. coli* O157:H7, *S. aureus* and *L. monocytogenes* using similar steps. Inhibition zone was linked to the bacteriocin compounds not LAB. The inhibition zone was measured using caliper.

2.8. Isolation and characterization of the 16S rRNA of lactic acid bacteria

Identification was carried out using PCR amplification of the 16s rRNA gene using primers 8F (AGA GTT TGA TCM TGG CTC AG) and 15R (AAG GAG GTG ATC CAR CCG CA) for 30 cycles as well as genome sequencing. The PCR amplification results were purified and processed using BioEdit Software. The LAB was identified using NCBI BLASTN at https://www.ncbi.nlm.nih.gov/. Differentiation used a set of four *rec*A gene-based primers of para F (GTC ACA GGC ATT ACG AAA AC), pants (CAG TGG CGC GGT TGA TAT C), plant (50-CCG TTT ATG CGG AAC ACC TA-30) and pREV (TCG GGA TTA CCA AAC ATC AC). The PCR product from the multiplex PCR was electrophoresed in 2% (w v^{-1}) agarose gels 1% TAE [15].

2.9. BLAST and phylogenetic analysis

Phylogenetic analysis was carried out based on the published methods [16]. Sequencing data were assembled using BioEdit Software, converted into FASTA format and then analyzed using BLAST at http://www.ncbi.nlm.nih.gov/blast/cgi. Phylogenetic analysis was carried out by inserting DNA sequences into the Clustal W2 at http://www.ebi.ac.uk/Tools/clustalw2/.

3. Results and Discussion

3.1. Total colonies of lactic acid bacteria from Bilih fish

Colony calculation was carried out to find out the number of LAB colonies using Quebec colony calculation formula. Presence of LAB in Bilih fish includes positive effects on consumers, including health benefits, especially in the digestive tract. The total number of LAB colonies is listed in Table 2.

Table 2. Total colonies of LAB in Bilih fish (*Mystacoleucus padangensis*) Singkarak Lake

Sampling location	Sample	Total of LAB ($\times 10^6$ CFU g^{-1})
Nagari Guguak Malalo	IB 1	64 ±0.04
Nagari Simawang	IB 2	27 ±0.01
Nagari Singkarak	IB 3	44 ±0.02

After LAB were cultured on MRS agar media, calculation was carried out to obtain the total LAB colony of Bilih fish in Singkarak Lake. The total LAB colony of Bilih fish is seen in Figure 3.

If the number of LAB colonies in fermented Bilih fish was high, this fish of Singkarak Lake was then better valued. The total LAB colony from isolate IB1 was 64×10^6 CFU g^{-1}, the total LAB colony from IB2 was 27×10^6 CFU g^{-1} and the total LAB colony from isolate IB2 was 44×10^6 CFU g^{-1}. Results showed that the highest total LAB colony from IB1 was 64×10^6 CFU g^{-1} [17], compared with the study on selar fish sauce, which was 2.3×10^2 to 1.35×10^4 CFU/g [18]. The total LAB colonies on tilapia were in the range of 8.83–10^6 CFU g^{-1}; higher than the study of Wizna [18], who reported the total LAB colony in the range of 10^6-10^9 CFU g^{-1} LAB and 4.76×10^6 to 5.30×10^8 CFU g^{-1} in Tilapia. Studies by Purwati reported total LAB in the range of 8.83×10^8 CFU g^{-1} [19]. Differences in the total number of LAB colonies were allegedly due to the effects of environmental conditions in the sampling locations, where the sampling location of IB1 (Nagari Guguak Malalo) included a low population density and did not include a traditional market, compared to the sampling locations of IB2 and IB3. Population density and traditional market in the sampling locations caused pollution in the Bilih fish ecosystem, where the community disposed of their household and market waste directly into the Singkarak Lake. Life of the microorganisms is highly dependent on and affected by the environmental conditions. However, the number of LAB colonies of Bilih fish from Singkarak Lake has met the Food and Agriculture Organization/World Health Organization (FAO/WHO) 2003 criteria because the probiotic foods included 10^6-10^8 CFU g^{-1} LAB. Technically, yellowish white was the color of LAB on MRS agar. These results were similar to those of a study carried out by Purwati, which produced yellowish-white LAB colonies on MRS agar [19]. Diversity in the number of colonies from Bilih fish LAB isolates was due to various morphologies, types of glucose fermentation, growth temperatures and nutritional conditions. These differences in the LAB ecosystem produced highly variable LAB isolates [20].

3.2. Antimicrobial activity of the lactic acid bacteria

Antimicrobial activity was carried out to assess if LAB were able to inhibit the growth of pathogenic bacteria. The pathogenic bacteria in this study included *E. coli* O157:H7, *S. aureus* ATCC 25923 and *L. monocytogenes* CFS-AN004330. *S. aureus* ATCC 25923 and *E. coli* O157:H7 are pathogenic bacteria that cause diseases in the digestive tract, while *L. monocytogenes* CFSAN 004330 is commonly found in foods stored at low temperatures. Screening used the diffusion well method. Diameters of the clear zones for the LAB isolate are listed in Table 3.

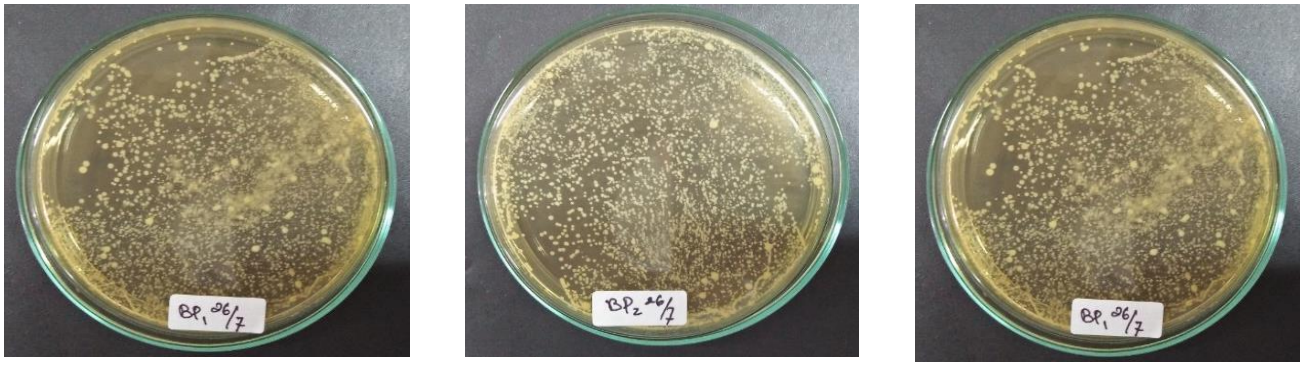
Table 3. Diameter of clear zone of antimicrobial activity test (mm)

No.	LAB Isolated	Clear zone diameter (mm)		
		Escherichia coli O157:H7	*Staphylococcus aureus*	*Listeria monocytogenes*
1	IB 1	27.29 ±0.03	14.17 ±0.01	11.14 ±0.01
2	IB 2	15.16± 0.03	12.09 ±0.02	9.16 ±0.03
3	IB 3	16.24± 0.05	10.18 ±0.05	10.04 ±0.08

Note: The value is expressed as the mean ±standard deviation; n=3

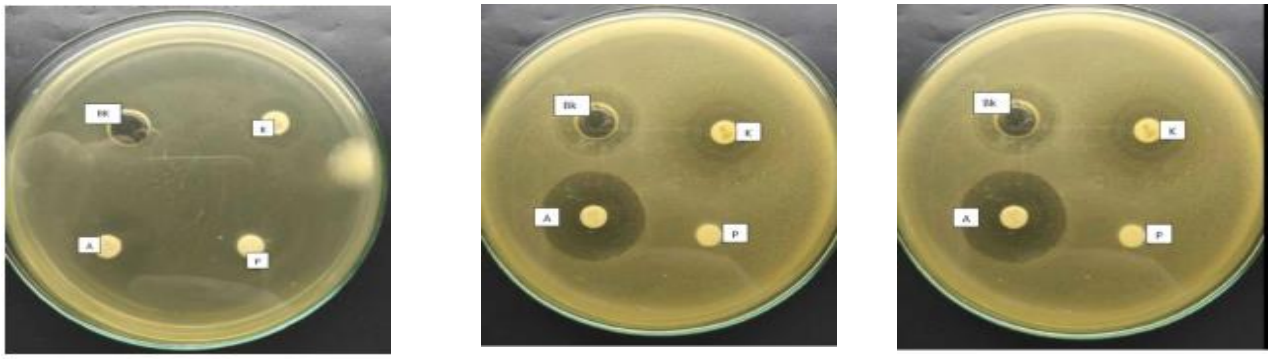
The largest inhibition zone formed on the *E. coli* O157:H7 culture belonged to IB1 with a diameter of 27.29 mm and the lowest inhibition zone belonged to IB2 with a diameter of 15.16 mm. Furthermore, the diameter of the largest inhibition zone against *S. aureus* ATCC 25923 belonged to IB1 of 14.17 mm and the lowest inhibition zone was linked to IB3 with a large diameter of 10.18 mm. The largest inhibition zone for *L. monocytogenes* CFSAN 004330 belonged to IB1 with a diameter of 11.14 mm while the lowest inhibition zone was linked to IB2 with a diameter of 9.16 mm. Inhibition zones of the LAB isolate against the three pathogenic bacteria can be seen in Figure 4.

The LAB isolates of IB1 included the largest inhibition zone with 27.29 mm in diameter against *E. coli* O157:H7, 14.17 mm in diameter against *S. aureus* ATCC 25923 and 11.14 mm in diameter against *L. monocytogenes* CFSAN0043. Isolate IB1 was used in antimicrobial inhibition assessments against pathogenic bacteria using penicillin, kanamycin and ampicillin as positive controls.



IB 1 IB 2 IB 3

Figure 3. LAB colony of Bilih fish on MRS Agar (IB1) sample from Nagari Guguk Malalo, (IB 2) sample from Nagari Simawang, (IB 3) sample from Nagari Singkarak



A B C

Figure 4. Antimicrobial activity test of LAB and antibiotic test Notes: (A) Clear zone formed against bacteria *Escherichia coli 0157* (B) Clear zone formed against *Staphylococcus aureus* bacteria (C) Clear zone formed against *Listeria monocytogenes* bacteria

This is based on the opinion of experts who state that penicillin is an antibiotic that can fight Gram-positive bacteria by inhibiting the synthesis of bacterial cell walls, including the beta-lactam group [21]. Ampicillins are penicillin derivatives that include a further broad-spectrum capable of acting against Gram-positive and Gram-negative bacteria. Furthermore, kanamycin belongs to the bactericidal aminoglycoside group, which usually acts against Gram-positive bacteria that cause infections. Antibiotics were administered using paper disks containing fixed concentrations of ampicillin 2 g g^{-1}, kanamycin 30 g g^{-1} and penicillin 3 g g^{-1}. To assess resistance and sensitivity of the bacteria, positive controls of the antibiotics were used. Results of the antimicrobial activity assay that of LAB isolates from Bilih fish of Singkarak Lake IB1 are shown in Table 4.

Based on the results of the study in Figure 4 and Table 4, it can be seen that the LAB IB1 isolate from Bilih fish was able to inhibit the growth of the three pathogens.

Table 4. Antimicrobial activity of LAB IB1 and antibiotic test

Sample Code	Inhibition zone (mm)		
	Escherichia coli O157:H7	*Staphylococcus aureus ATCC 25923*	*Listeria monocytogenes*
IB1	27.29 ±0.03	14.17 ±0.01	11.14 ±0.01
Penicillin	-	-	-
Ampicillin	22.28 ±0.05	29.35 ±0.02	11.14 ±0.05
Kanamycin	21.26 ±0.09	22.29 ±0.05	14.19 ±0.08

L. monocytogenes CFSAN0043 forms a diameter of 11.44 mm which is high compared to the results of the study by Ramos, which assessed the antimicrobial activity of the traditional Dadih food; in which, *L. monocytogenes* CFSAN 0043 was used and produced a 9 mm clear zone [22]. In this study, *E. coli* O157 caused a clear zone with a diameter of 27.29 mm which was higher than the results of previous studies by Chelule regarding the antimicrobial activity of LAB isolated from soybean shells on *E. coli* O157 with a clear or inhibition zone of 8.31 mm [23]. The *S. aureus* ATCC 25923 formed a clear zone of 14.17 mm. The results of this study are relatively higher than the results of studies that have been found by Anggraini regarding the antimicrobial activity of LAB isolates from okara against the pathogenic *S. aureus* with a 9.10-mm clear zone [24]. In the study of Galvez, a larger clear zone diameter against pathogenic bacteria was reported, ranging 22-32.5 mm [25]. Differences in the results were due to the LAB isolates from various samples. In this study, samples of Bilih fish isolates from Singkarak Lake were used, different from the results of studies that used isolated from fermented Budu fish [25]. Moreover, when compared with several previous studies that the diameter of the inhibition zone against S. aureus formed was 9.35-10.22 mm [26], while these were 10.38-11.06 mm against *E. coli* [27]. Their study showed that diameters of the clear zones formed by *L. plantarum* against *E. coli*, *S. aureus* and *Enterococcus faecalis* were 13.75, 16.75 and 15.4 mm, respectively. Detected that the diameter of the inhibition zone of *Lactococcus* for several test bacteria was smaller, ranging 0-15 mm. Results showed that the LAB isolates from Bilih fish from Singkarak Lake were able to inhibit the growth of pathogenic bacteria that could harm humans, especially *L. monocytogenes*. These bacteria cause abortion in humans or livestock. The LAB could be used as a treatment based on the zone of inhibition tested on pathogenic bacteria. The inhibitory activity was divided into four categories, weak activity (< 5 mm), moderate (5-10 mm), strong (> 10-20 mm) and very strong (> 20-30 mm). Therefore, it revealed that the LAB IB1 from Bilih fish of Singkarak Lake included strong activity on the three bacteria**.** The main role of lactic acid bacteria is to ferment carbohydrates to produce organic acids, which can cause a decrease in pH. The low pH and the presence of organic acids, especially lactic acid, are the main factors in the preservation process of fermented fish products. Generally, pH between 4.5 and 5.0 can inhibit pathogenic bacteria and decomposers [28].

3.3. Antimicrobial activity of the raw bacteriocin supernatant

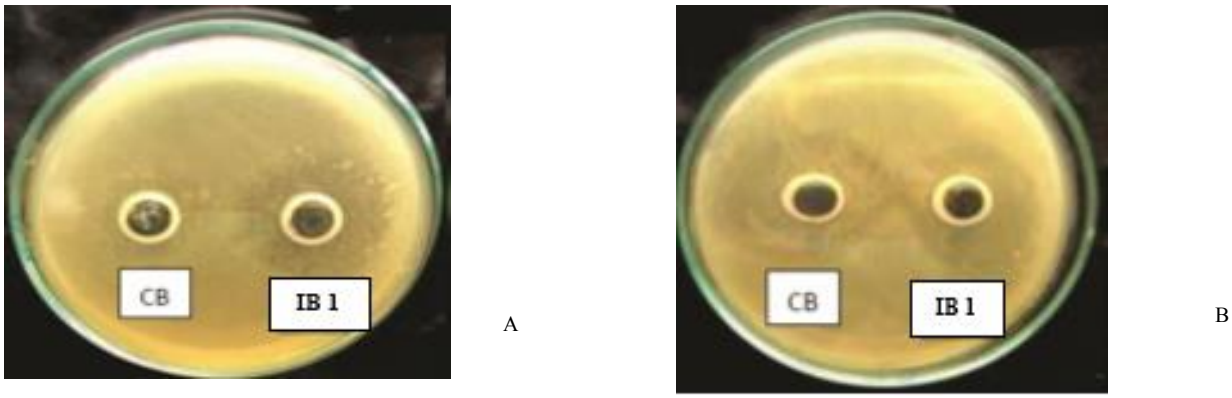
Assessment of the antimicrobial activity of the crude bacteriocin supernatant from Bilih fish isolates was carried out after neutralizing pH of the LAB supernatant; thus, no antimicrobial activity of organic acids occurred. Organic acid components, especially lactic acid, were the major components of antimicrobial compounds for LAB [28]. Table 5 shows antimicrobial activity of the neutralized supernatant against *E. coli* O157:H7 and *S. aureus* ATCC 25923; however, activity on *L. monocytogenes* is not shown.

Table 5. Antimicrobial activity of the raw bacteriocin supernatant from Bilih fish of Singkarak Lake, West Sumatera, Indonesia

Pathogenic bacteria	Inhibition zone diameter (mm)
Escherichia coli O157:H7	16.89 ±0.02
Staphylococcus aureus ATCC 25923	20.79 ±0.01
Listeria monocytogenes CFSAN004330	17.19 ±0.01

Note: The value is expressed as the mean ±standard deviation; n=3

Based on the results in Table 5 and Figure 5, the activity was higher compared with that of Melia, where the study discussed the crude bacteriocin activity of LAB isolates from vinegar against *S. aureus* ATCC 25923 with 13.1. mm of clearance and against *E. coli* O157:H7 with 12.7 mm of clearance [29].



A B

Figure 5. Antimicrobial activity of the crude bacteriocin after neutralizing pH (CB) and before neutralizing pH (IB1) against *Escherichia coli* O157:H7 (A) and *Staphylococcus aureus* ATCC 25923 (B)

However, a study reported that LAB isolates did not include antimicrobial activity after the pH of the supernatant was neutralized; hence, it was concluded that the antimicrobial activity was originated from organic acid compounds produced by the LAB [30]. Furthermore, *L. Plantarum* NS (9) from isolates derived from tilapia as food for fermented fish products from South Sumatra [31]. It was reported that antimicrobial activity was linked to the organic acid compounds. The highest antibacterial activity against *E. coli*, *B. cereus* and *L. monocytogenes* was reported at the end of the exponential growth phase (12-15 h incubation time) while the highest antibacterial activity against *S. aureus* and *S. typhimurium* ATCC 14028 was seen at 21 and 24-h incubation time. Furthermore, several research results have revealed that the antimicrobial activity of cell-free cultures of *L. plantarum* and *L. brevis* supernatants isolated from fermented fish meat (bekasam) in South Sumatra was able to inhibit E. coli and L. monocytogenes [32]. Found that Weissellicin 110, a class II bacteriocin produced by Weissellicin 110 isolated from pla som, was able to inhibit Gram-positive bacteria with no antimicrobial activity against *L. monocytogenes* [33]. Crude bacteriocins from LAB were isolated from Chao and assessed against *S. aureus* FNCC 0047 and *E. coli* FNCC 0049. There were several mechanisms to inhibit the destruction of target cells by bacteriocins [33]. Inhibition of the formation of lipid II (cell wall precursor) can be carried out by the following mechanism of a) inhibition of cell wall biosynthesis and b) stabilization of the formation of membrane target pores. In fact, when the peptide attaches to the target cell membrane, the positive end of the peptide binds to the fatty acid in the phospholipid layer of the target bacterial membrane. This step involves binding of the peptide to a membrane-like monomer, resulting in a separation that leads to the formation of pores and eventual cell death [34] .

3.4. Identification of the lactic acid bacteria from Bilih fish of Singkarak Lake using 16S rRNA

3.4.1. The 16S rRNA gene amplification using polymerase chain reaction

Electrophoresis results showed that the PCR was successful in amplifying the bacterial 16S rRNA gene from the LAB isolates of Bilih fish. This was verified by fragments of the PCR product with sizes of 1529 bp, using 27F AGAGTTTGATCCTGGCTGAG primer for the forward and 1429 R GTTTACCTTACGACTT primer for the reverse directions. Results of the amplification of 16S rRNA gene of the LAB from Bilih fish were used to ensure the success of the genomic DNA isolation process. Furthermore, results from the 16S rRNA gene amplification was used to identify LAB [35]. Amplification of the 16S rRNA gene was followed by nucleotide sequencing. Electrophoresis results of the PCR products from LAB isolates are shown in Figure 6.

The aim of molecular approach was to analyze genetic relationships of the LAB by comparing ribosomal RNA sequences. As a relatively small molecule, 16S rRNA can be sequenced directly without the need of cloning PCR amplicons [35]. Figure 6 shows results of DNA amplification of 1429-bp fragments on agarose gel. This indicated that the specific primer used in the study was able to identify bacteria to the strain level.

3.4.2. Sequence analysis of the 16S rRNA gene in isolates from Bilih fish (*Mystacoleucus padangensis*) of Singkarak Lake

Results from the bacterial sequencing of IB1 were compared with data on GenBank, using Basic Local Alignment Search Tool (BLAST) and sequence homology (http://www.ncbi.nlm.nih.gov).

Based on the data analysis (Figure 7) using BLAST, IB1 from Bilih fish of Singkarak Lake included a high similarity value of 99.61% that was close to that of *L. fermentum* Strain 17059 16S. This demonstrated that LAB in Bilih fish of Singkarak Lake were close to *L. fermentum* Strain 17059 16S according to a study of Mustarim, which stated that the isolates with more than 97% similarity of 16S rRNA sequences could represent a same species while sequence similarities of 93-97% could represent bacteria at the genus level with various species [36]. There is only a 3% difference in sequence of the 16S rRNA gene or a sequence homolog of 97%, a sequence homolog with a value of 97% is equivalent to a minimum number of 70% hybridization used to express two bacteria belonged to one species [36]. Results of the BLAST analysis from this study are shown in Table 6.

Based on the BLAST results in Table 6, it shows that IB1 included a high similarity of 99.61% to strains of *Lactobacillus* spp. Experts state that a sequence can be categorized as homologous if the sequence has more than 70% similarity [36]. The bacterial relationship analysis in BLAST was then visualized using MEGA Software v7.0 and alignments from BioEdit Software. From the results of phylogenetic visualization, it can be seen that the isolates of Bilih fish of Singkarak Lake in this study included apomorphic relationships with *L. fermentum* Strain 17059 16S [37], revealing that the characters were apomorphic and plesiomorphic. Apomorphic characters are characters that can be changed, inherited and detected in groups, while synapomorphic characters are characters that can be inherited and occurred in monophyletic groups.

Based on the PCR results and after being analyzed using BLAST and based on the phylogenetic tree in Figure 8, it is known that the IB1 bacterial isolate from the Lake Singkarak Bilih fish has a 99.61% similarity with L *L. fermentum* Strain 12042 16S. A sequence is categorized as homologous if it includes more than 70% of similarity. The phylogenetic tree showed close relationships between the *L. fermentum* strains [39].

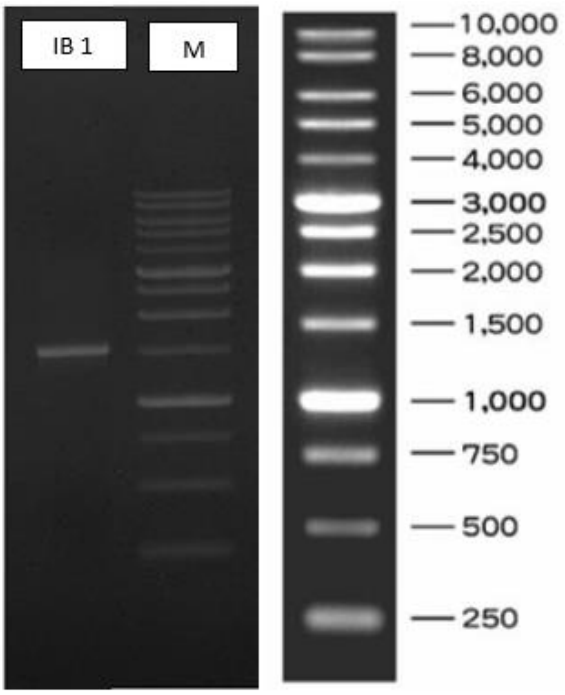


Figure 6. Gel electrophoresis on the polymerase chain reaction products of the lactic acid bacteria from Bilih fish. M, DNA ladder; and IB1, LAB isolate from the Bilih fish of Singkarak Lake, West Sumatera, Indonesia

Table 6. The BLAST results of the lactic acid bacteria (IB1) from Bilih fish of Singkarak Lake, West Sumatera, Indonesia

No	Description of *Lactobacillus fermentum* strain	Query cover (%)	Accession number	Percent identification (%)
1	L1 16S chromosome		CP076446.1	
2	17059 16S		MW866783.1	
3	19478 16S		MW674525.1	
4	100716S chromosome		CP071001.1	
5	GR 1103 16S chromosome		CP070858.1	
6	16425 16S		MW486795.1	
7	16096 16S		MW479248.1	
8	16004 16S	100	MW475239.1	99.61
9	15077 16S		MW463597.1	
10	14924 16S		MW463534.1	
11	ban2-VX-D14-6 16S		MT903054.1	
12	9323 16S		MT845951.1	
13	9321 16S		MT845949.1	
14	9138 16S		MT810093.1	
15	9136 16S		MT810091.1	

Sequence Assembly

>CONTIQ_IB1
AAAGATGGCTTCTCGCTATCACTTCTGGATGGACCTGCGGTGCATTAGCTTGTTGGTG
GGGTAATGGCCTACCAAGGCGATGATGCATAGCCGAGTTGAGAGACTGATCGGCCAC
AATGGGACTGAGACACGGCCCATACTCCTACGGGAGGCAGCAGTAGGAATCTTCCAC
AATGGGCGCAAGCCTGATGGAGCAACACCGCGTGAGTGAAGAAGGGTTTCGGCTCGT
AAAGCTCTGTTGTTAAAGAAGAACACGTATGAGAGTAACTGTTCATACGTTGACGGTA
TTTAACCAGAAAGTCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGG
CAAGCGTTATCCGGATTTATTGGGCGTAAAGAGAGTGCAGGCGGTTTTCTAAGTCTGA
TGTGAAAGCCTTCGGCTTAACCGGAGAAGTGCATCGGAAACTGGATAACTTGAGTGC
AGAAGAGGGTAGTGGAACTCCATGTGTAGCGGTGGAATGCGTAGATATATGGAAGAA
CACCAGTGGCGAAGGCGGCTACCTGGTCTGCAACTGACGCTGAGACTCGAAAGCATG
GGTAGCGAACAGGATTAGATACCCTGGTAGTCCATGCCGTAAACGATGAGTGCTAGG
TGTTGGAGGGTTTCCGCCCTTCAGTGCCGGAGCTAACGCATTAAGCACTCCGCCTGGG
GAGTACGACCGCAAGGTTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTG
GAGCATGTGGTTTAATTCGAAGCTACGCGAAGAACCTTACCAGGTCTTGACATCTTGC
GCCAACCCTAGAGATAGGGCGTTTCCTTCGGGAACGCAATGACAGGTGGTGCATGGT
CGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCCGCAACCCTT
GTTACTAGTTGCCAGCATTAATTTGGGCACTCTAGTGAGACTGCCGGTGACAACCGGA
GGAAGGTGGGGACGACGTCAGATCATCATGCCCCTTATGACCTGGGCT

Figure 7. Nucleotide sequencing of the lactic acid bacteria (IB1) from Bilih fish of Singkarak Lake, West Sumatera, Indonesia

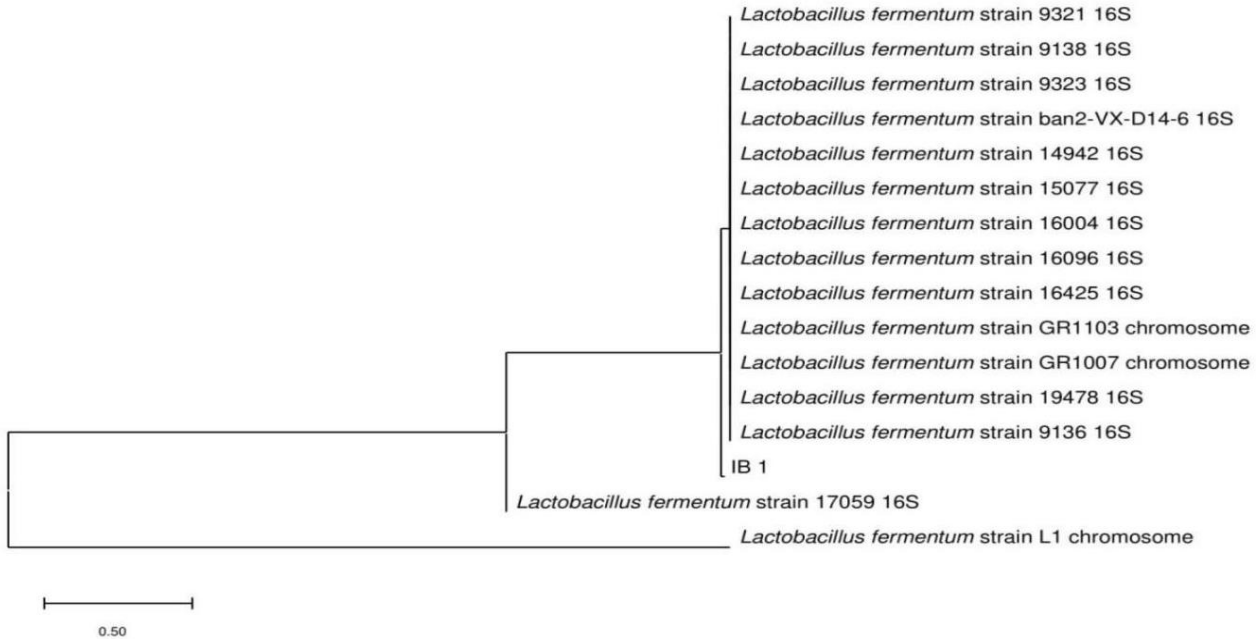


Figure 8. Phylogenetic tree construction of the isolates (IB1) from Bilih fish of Singkarak Lake, West Sumatera, Indonesia, using neighbor-joining method, Kimura 2 model and MEGA v.7.0

A clear pattern was demonstrated; in which, the bacterial formed monophyletic groups with *L. fermentum* 12042 16S. This indicated reliability of close relationships between bacteria from a common ancestor [39]. Results of this study vary from those reported by Tjong on isolates of *Weisella paramesenroides* from budu fish from West Pasaman Regency [40], while *Pediococcus pentosaceus* species from Congkok Fish from 50 Kota area [41]. Isolation of LAB in curd samples reported 36 strains of *Lactobacillus* and *Streptococcus* Sp. [42]. Some research reported *L. casei* as the dominant LAB in fermented budu fish from Padang Pariaman Regency, which varied because processes of making fish fermented in each region varied; thus, the bacteria could also include various species [43]. This is supported by Melia that Bilih fish meal of Singkarak Lake in West Sumatera is made from rice as the basic ingredient by relying on microorganisms that naturally exist as inoculants without using additional starters [44]. Microorganisms are known to be derived from banana leaves as the cover of the containers and from the fish as well as from the rice [44].

4. Conclusion

Results from the molecular identification of 16S rRNA showed antimicrobial potentials of the LAB as isolates from

Bilih fish (Singkarak Lake, West Sumatra, Indonesia). *L. fermentum* Strain 17059 16S is a novel strain in Bilih fish. Similar to *L. fermentum* Strain 12042 16S, the current isolated species can be used as an anti-diarrhea and anti-typhoid LAB candidate in humans and as a natural food preservative. However, further assessments on the bacteriocins produced by the LAB from Bilih fish of Singkarak Lake are necessary, including bacteriocin characterization for the food biopreservation.

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6. Conflict of Interest

The authors report no conflicts of interest.

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پتانسیل ضد میکروبی *لاکتوباسیلوس فرمنتوم* جدا شده از ماهی بیلیه (Mystacoleucus padangensis) دریاچه سینگکاراک، سوماترا غربی، اندونزی

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- ضدمیکروبی
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- باکتری‌های لاکتیک اسید
- ماهی بیلیه

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چکیده

سابقه و هدف: باکتری‌های لاکتیک اسید اخیراً یکی از موضوعات اصلی بحث در زمینه‌های بهداشت، صنایع غذایی، علوم، دامپروری و کشاورزی شده‌اند. باکتری‌های لاکتیک اسید به‌طور گسترده‌ای در تخمیر انواع گوناگون فرآورده‌های غذایی تهیه شده از حیوانات، ماهی‌ها و گیاهان استفاده می شود که به‌عنوان نگهدارنده عمل می‌کنند و اثرات مثبتی بر سلامت و زیبایی انسان دارند. یکی از منابع باکتری لاکتیک اسید ماهی *Mystacoleucus padangensis* دریاچه سینگکاراک، در سوماترا غربی اندونزی است جایی‌که در آن یک باکتری زیست‌یار[۱] از *لاکتوباسیلوس فرمنتوم* با پتانسیل ضد میکروبی جدا شده است. هدف از این مطالعه بررسی پتانسیل ضد میکروبی *لاکتوباسیلوس فرمنتوم* جدا شده از ماهی بیلیه از دریاچه سینگکاراک اندونزی بود.

مواد و روش ها: روش‌های این مطالعه جداسازی باکتری‌های لاکتیک اسید از ماهی بیلیه از دریاچه سینگکاراک و متعاقب آن ارزیابی فعالیت ضد میکروبی روماند[۲] باکتریوسین خام بود. سپس از S rRNA۱۶ برای ارزیابی گونه‌های جدا شده باکتری لاکتیک اسید استفاده شد. از سه نمونه، یک ایزوله IB1 با پتانسیل فعالیت ضدمیکروبی گزارش شد.

یافته‌ها و نتیجه‌گیری: یافته‌های مطالعه نشان داد که ویژگی‌های مورفولوژیکی و بیوشیمیایی باکتری‌های لاکتیک اسید گرم مثبت، باسیل شکل و کاتالاز منفی از گروه باکتری‌های جور تخمیر[۳] می‌باشد. بیشترین فعالیت ضد میکروبی توسط IB1/*شرشیا کلی* 0157:H7 (۲۷/۲۹ mm)، *استافیلوکوکوس اورئوس* ATCC 25923 (۱۴/۱۷ mm)، و *لیستریا مونوسیتوژنز* CFSAN 004330 (۱۱/۴۴ mm) نشان داده شد، در حالی‌که قطر ناحیه مهاری رشد رومایه باکتریوسین خام باکتری‌های لاکتیک اسید *شرشیا کلی* 0157:H7 ۱۶/۸۹mm بود *استافیلوکوکوس اورئوس* ATCC 25923 ناحیه مهار رشد ایجاد نکرد. در pH خنثی برای *لیستریا مونوسیتوژنز* CFSAN 004330 فعالیت ضد میکروبی مشاهده نشد. نتایج شناسایی مولکولی با استفاده از S rRNA۱۶ نشان داد که باکتری لاکتیک اسید جدا شده شباهت‌هایی با *لاکتوباسیلوس فرمنتوم* سویه 16S 17059 دارد، شامل پتانسیل ضد میکروبی در برابر باکتری‌های بیماری‌زا می‌باشد. *لاکتوباسیلوس فرمنتوم* سویه 16S 17059 می‌تواند به‌عنوان یک عامل ضد اسهال و ضد تیفوئید در انسان و به‌عنوان یک نگهدارنده طبیعی در مواد غذایی مورد استفاده قرار گیرد.

تعارض منافع: نویسندگان اعلام می‌کنند که هیچ نوع تعارض منافعی مرتبط با انتشار این مقاله ندارند.

[۱] probiotic bacterium
[۲] supernatant
[۳] homofermentative