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Isolation and Characterization of Lactic Acid Bacteria (LAB) With Potential As Probiotics from Shrimp Paste Tanjungbalai Asahan

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ABSTRACT
Terasi or belacan is an example of a fermented product from shrimp that involves lactic acid bacteria. Lactic acid bacteria are a group of gram-positive bacteria that have the ability to produce antagonistic molecules that can be used as antibacterial probiotics and preservatives. This study was conducted to determine the presence of lactic acid bacteria which might detect shrimp paste in Tanjungbalai and to the antibacterial activity. This study uses descriptive data analysis, which begins with isolation and characterization by carrying out gram staining and biochemical tests and then continuing with a probiotic potency test which includes a test to acidity (pH) and antibacterial activity test of phatogens. Isolations and characterization of LAB produced 6 isolates in the form of coccus, the edges of the colony were whole or flat, the elevation was flat or milky white in color. Meanwhile, the cell morphology of 6 LAB isolates was in the form of bacilli, gram positive and monobacillary arrangement. Characterization based on biochemical tests found isolates belonging to the genus Pediococcus, Weisella and Lactobacillus. The results of probiotic tests of 6 isolates were expressed with gastric acid conditions (pH 2.5-3) and had phatogen antibacterial activity which was marked by the formation of an inhibition against 2 Escherichia coli test bacteria with the largest inhibition zone size being 8.95 mm while Staphylococcus aureus with the largest inhibition zone size was 12.6 mm. Based on the research that has been done, it can be concluded that LAB from Tanjungbalai shrimp paste has potential as a probiotic because the six isolates obtained met the requirements of bacteria as probiotics, namely being able to withstand acidic conditions and having phatogenic antibacterial activity.

Keyword: Lactic Acid Bacteria, Probiotics, Shrimp paste.

ABSTRAK
Terasi atau belacan merupakan contoh produk fermentasi dari udang yang melibatkan bakteri asam laktat. Bakteri Asam Laktat merupakan suatu kelompok bakteri gram positif yang memiliki kemampuan menghasilkan molekul antagonis yang dapat dimanfaatkan sebagai probiotik antibakteri dan pengawet. Penelitian ini dilakukan untuk mengetahui adanya Bakteri Asam Laktat (BAL) yang berpotensi sebagai probiotik pada terasi udang di Tanjungbalai Asahan dan adanya aktivitas antibakterinya. Penelitian ini menggunakan analisis data deskriptif yang diawali dengan isolasi dan karakterisasi dengan melakukan pewarnaan gram serta uji biokimia lalu dilanjutkan uji potensi probiotik yang meliputi uji ketahanan terhadap keasaman (pH) dan uji aktivitas antibakteri patogen. Hasil Isolasi dan karakterisasi menghasilkan 6 isolat yang teridentifikasi berbentuk Coccus, tepi koloni berbentuk entire, elevasi berbentuk Flat yang berwarna putih susu.

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Sedangkan morfologi sel 6 isolat BAL teridentifikasi berbentuk basil, gram positif dan penataan monobasil. Karakterisasi berdasarkan uji biokimia didapati keenam isolat tergolong genus Pediococcus, Weisella dan Lactobacillus. Hasil uji probiotik 6 isolat dinyatakan positif terhadap kondisi asam lambung (pH 2.5-3) dan keenam isolat BAL asal terasi Tanjungbalai Asahan memiliki aktivitas antibakteri terhadap bakteri uji Escherichia coli dan Staphylococcus aureus dengan kekuatan daya hambat yang dikategorikan kuat pada 1 isolat (NT1) dengan rata-rata ukuran zona hambat 12.6 mm pada bakteri uji S. aureus dan 8.95 mm pada bakteri uji E. coli sedangkan kelima isolat lainnya memiliki kekuatan daya hambat yang dikategorikan lemah dengan rata-rata ukuran zona hambat 0.7 mm – 2.5 mm.

Kata kunci: *Bakteri Asam Laktat, Probiotik, Terasi.*

1. Introduction

Terasi is a traditional fermented shrimp paste from Indonesia that is frequently used as a condiment to improve the flavor of Indonesian dishes [1]. Stated that terasi is a preserved product of small fish or rebon that has been processed through fermentation, grinding or pounding and drying. Terasi or better known by the people of Tanjungbalai Asahan often called belacan is one example of a fermented product from shrimp or fish [2]. Based on observations made to consumers of shrimp paste in Tanjungbalai, it is reported that the distinctive taste of shrimp paste from Tanjungbalai Asahan is the most common type of shrimp paste.

Lactic acid bacteria have an important role in the process of making shrimp paste because they are able to produce organic acids that not only cause the taste of shrimp paste to be distinctive but can also produce substrates with low pH so as to prevent the growth of spoilage microorganisms [3]. Antagonistic molecules produced by Lactic acid bacteria are bacteriocins which are antibacterial peptides. Terasi is one of the preparations from the fermentation process made from fish and shrimp with salting process. The addition of high salt in the fermentation process can result in the growth of lactic acid bacteria. In fermented products, it is known that there are many lactic acid bacteria that are utilized as probiotics [4]. Probiotics are defined as live mycoorganisms that if consumed in a balanced or sufficient amount will provide health benefits.[5]

Isolation of Lactic acid bacteria from shrimp paste was conducted to determine its potential as a probiotic candidate and to see its ability to prevent the growth of pathogenic bacteria. There are several previous studies that have also isolated lactic acid bacteria in shrimp paste, including research conducted by Munaroh where he obtained 6 probiotic LAB isolates from shrimp paste from Yogyakarta, some of which have been identified as Lactobacillus casei subs. Rhamnishus, one isolate of Lactobacillus plantarum and three isolates of Pediococcus acidilactici [6] In addition, there is also research by Haitham from 166 LAB isolates from Malaysian belacan, there are 13 bacterial isolates that show gastrointestinal resistance and are able to inhibit unwanted bacteria [7].

Lactic acid bacteria that have the potential as pathogenic antibacterial probiotics are necessary to determine the quality of the resulting product. The higher the inhibition power produced from LAB from shrimp paste will show the better quality of shrimp paste obtained, because LAB is able to suppress the growth rate of pathogenic bacteria. This research will isolate Lactic acid bacteria in 3 shrimp paste products in Tanjungbalai Asahan. Therefore, researchers are interested in examining the isolation and characterization of Lactic acid bacteria that have the potential as probiotics from shrimp paste in Tanjungbalai Asahan.

2. Methods

2.1 Time and Place Research

This research was carried out for 3 months at the Microbiology laboratory, Biology, Faculty of Mathematics and Natural Sciences (FMIPA), The Universitas Sumatera Utara (USU), Medan, North Sumatra.

2.2 Tools and Materials

This research will use several tools such as petri dishes, pyrex, bunsen, hot plate, erlenmeyer, measuring cup, analytical balance, bio freezer-80℃, incubator 37℃, oven, micropipette, waterbath, stirrer plate, ultracentrifuge temperature 4℃, spectrophotometer, phase contrast microscope, microcentrifuge, pH meter. The materials uses in this research such as samples of shrimp paste (belacan), 70% alcohol, safranin, spiritus,

agar media. In the antibacterial activity test, this study used 2 test bacteria, namely *Escherichia coli* and *Staphylococcus aureus*. Chemicals used, NaCl, HCl 0.1 N, NaOH, H2SO3.

2.3 Isolation of Bacteria

The initial process of isolation is after diluting to 10-3, taken using a sterile volume pipette 1 ml sample from a 10-3 dilution, then inoculated by the pour plate method on MRSA (de Man Rogosa Sharpe Agar) media into a Petri dish. After that, it was incubated for 3 x 24 hours at a temperature of 37℃ . All growing colonies will be purified on other MRSA media using the streak plate method in order to obtain the desired pure isolate. Purification results will be incubated at 37℃ for 3 days.

2.4 Characterization of Bacteria (Gram staining)

1 ose of bacterial isolate was taken and then applied to the surface of the glass object sufficiently and fixed several times over a bunsen burner flame. Then given 2 drops of crystal violet and left for 60 seconds. Then washed using distilled water and dried. Next, the isolate was given a drop of iodine, left for 60 seconds then washed again with distilled water and dried. Next, the bacterial isolate was slowly dripped with 95% alcohol for 30 seconds. Then washed again with distilled water and dried. The last stage, the isolate was stained with safranin solution for 30 then washed with distilled water and dried. Bacterial isolates that have been given coloring are then observed under a microscope with a magnification of 100x.

2.5 Biochemical Test of Bacteria

Biochemical tests performed in this study include the Triple Sugar Iron Agar (TSIA) test, motility test, citrate test and catalase test.

2.6 Probiotic Test

a. Test for Resistance to Acidity (pH)

The acidity resistance test process begins with adding 0.1 N HCL solution to MRSB , isolate resistance to gastric acid conditions in the intestine with a low pH of 2.5-3. This test process begins with inoculating 1 round ose of isolate on MRSB HCL (Hydromedia and then incubated for 2 x 24 hours at 37℃. The test results are marked positive if there is bacterial growth on media that has a pH of 2.5-3, namely MRSB media becomes cloudy. If there is no growth on the media then the results can be declared negative [9].

b. Pathogenic Antibacterial Activity Test

Antibacterial activity tests were carried out by inserting bacterial isolates as much as 1 to 2 ose then incubated for 1 x 24 hours. Then centrifuge 12,000 rpm for 20 minutes and separated between the supernatant and the pellet. After that, the supernatant is heated to a temperature of 80℃ for 5 minutes. Then check the pH if it is too low, NaOH is added to neutralize it. The next process is prepared MHA media in a Petri dish then made a suspension of 2 test bacteria, namely *Escherichia coli* and *Staphylococcus aureus* with OD (Optical Dencity) 0.5. Then scratch the bacterial suspension using a cotton bud, using the quadrant method. Then the Petri dish was labeled with the sample code name. After that, the disc paper in the supernatant that has been centrifuged earlier is then placed into a Petri dish that has been given a scratch of the test bacterial suspension. Then incubated for 2 x 24 hours, then measured the clear zone formed using vernier caliper.

2.7 Data Analysis

The results of this research were statistic analyzed using One Way Analysis of Variance (ANOVA) SPSS version 20.

3. Result and discussion

3.1 Isolation and Characterization of Bacteria

Based on the research conducted, 6 LAB isolates were obtained from Tanjungbalai Asahan shrimp paste. Colony morphology which includes colony shape, colony edge, colony height and colony color, as well as cell morphology which includes cell shape and gram staining are used to characterize bacterial isolates (Table 3.1). colony shape in 6 isolates that have been identified is coccus-shaped while the colony edge is entire or flat. Elevation on the 6 isolates identified as flat or flat which is milky white in color. The purpose of characterization on cell morphology is to determine the shape of the isolate by performing gram staining. From the gram staining process that has been carried out, the results obtained are that all LAB isolates identified are gram-positive with bacillus or rod-shaped cells characterization that has been carried out on bacterial isolates isolated from shrimp paste from Jerowaru.District that all isolates have Gram-positive characters [10].

Table 3.1. Morphological Observation of Colonies and Cells of Bacteria

Colony Morphology					Cell Morphology		
Isolate	**Shape**	**Adge**	**Elevation**	**Color**	**Gram**	**Shape**	**Arrangement**
NT1	*Coccus*	Entire	Flat	Milky white	+	Basil	Diplobasil
NT2	*Coccus*	Entire	Flat	Milky white	+	Basil	Diplobasil
NT3	*Coccus*	Entire	Flat	Milky white	+	Basil	Monobasil
NT4	*Coccus*	Entire	Flat	Milky white	+	Basil	Streptobasil
NT5	*Coccus*	Entire	Flat	Milky white	+	Basil	Monobasil
NT6	*Coccus*	Entire	Flat	Milky white	+	Basil	Monobasil

3.2 Biochemical Test of Bacteria

The purpose of bacterial biochemical testing is to identify and determine a bacterial isolate through its physiological properties. Some biochemical tests that will be carried out on the 6 isolates in this study include the TSIA (Triple Sugar Iron Agar) test, citrate test, motility test and catalase test.

Table 3.2 Biochemical Test Results of Tanjungbalai Terasi

Isolate	TSIA Test	Citrate Test	Motility Test	Catalase Test	Genus
NT1	m/k (black sediment)	+	Non Motile	-	*Lactobacillus*
NT2	m/k	+	Non Motile	-	*Lactobacillus*
NT3	m/k	-	Non Motile	-	*Weisella*
NT4	m/k	+	Non Motile	-	*Pediococcus*
NT5	m/k	-	Non Motile	-	*Weisella*
NT6	m/k(black sediment)	+	Non Motile	-	*Pediococcus*

- m : red
- k : yellow
- \+ : positive (change occurs)
- \- : negative (no change)

Based on the results of biochemical tests that have been carried out, the six isolates can be classified into 3 genus of lactic acid bacteria namely *Pediococcus, Weisella* and *Lactobacillus* these bacteria are classified with reference to Bergey's Manual of Systematic Bacteriology Second Edition, volume three: The femicutes. *Pediococcus* is a genus of gram-positive bacteria that are part of bacteria, the main characteristic of these bacteria is that they can produce lactic acid and fermentation. These bacteria are also facultatively anaerobic, non-motile, citrate positive and unable to produce catalase enzymes. The genus *Lactobacillus* is classified as bacteria that have gram-positive characteristics, negative catalase, acid-tolerant, non-motile, positive in the citrate test and no spore. While the genus *Weissella* cells are short rods, classified as gram-positive bacteria, non-motile, facultative anaerobic, some bacteria are citrate positive and citrate negative, negative catalase and facultative anaerobic genus *Weissela* includes bacteria because of its metabolism and phylogenetic position.

3.3 Probiotic Test

The probiotic test in this study includes 2 tests, namely the acidity resistance test (pH) and the pathogenic antibacterial test (*E. coli* and *S. aureus*).

3.4 Test for Resistance to Acidity (pH)

The results of the acidity (pH) resistance test on the six LAB isolates from Tanjungbalai Asahan shrimp paste were positive. The six isolates were able to grow at pH 2.5-3. This research accodance with previous research conducted by Ansumar and Leni stated that 7 isolates of LAB from Riau shrimp paste showed positive results in the acidity (pH) resistance test [8] LAB is relatively tolerant of low pH. To be used as a probiotic, LAB must be able to survive at low pH, because stomach acidity ranges from pH 2.0-4.0 caused by HCl production.

Tabel 3.3 Test for Resistance to Acidity (pH)

Isolate	Resistance Test Acid (pH)
NT1	+
S	+
NT3	+
NT4	+
NT5	+
NT6	+

Result (+) : cloudy

3.5 Pathogenic Antibacterial Activity Test

Based on the tests that have been carried out, the results of the formation of a clear zone of LAB isolates of Tanjungbalai Asahan shrimp paste show that the six isolates have pathogenic antibacterial activity which is known because a clear zone has formed around the disc paper. The formation of a clear zone around the disc paper with a firm and clear circle border indicates the presence of antibacterial activity [11].

Table 3.4 Measurement Data of Zone of Inhibition of *Staphylococcus aureus* Test Bacteria

Isolate	***Staphylococcus aureus* (Gram Positive)**				Average	Criteria
	U1	U2	U3	U4		
NT1	13.6 mm	9.7 mm	14.3 mm	13.1 mm	12.6 mm	Strong
NT2	1 mm	3.2 mm	4.1 mm	1 mm	2.3 mm	Weak
NT3	0.7 mm	1 mm	0.5 mm	0.7 mm	0.7 mm	Weak
NT4	2.4 mm	0.6 mm	0.7 mm	0.6 mm	1.0 mm	Weak
NT5	0.8 mm	1 mm	1 mm	0.9 mm	0.9 mm	Weak
NT6	0.7 mm	0.7 mm	0.8 mm	0.5 mm	0.6 mm	Weak
Control (+)	21.9 mm	24 mm	25.5 mm	23.8 mm	23.8 mm	Very Strong
Control (-)	-	-	-	-	-	-

Table 3.5 Measurement Data of Zone of Inhibition of *Escherichia coli* Test Bacteria

Isolate	***Escherichia coli* (Gram negative)**				Average	Criteria
	U1	U2	U3	U4		
NT1	9.7 mm	9.3 mm	10.8 mm	6.0 mm	8.95 mm	Medium
NT2	1 mm	1 mm	4.5 mm	6.0 mm	3.12 mm	Weak
NT3	1.2 mm	0.9 mm	1.8 mm	2.5 mm	1.6 mm	Weak
NT4	2.5 mm	0.6 mm	2.4 mm	3.1 mm	2.1 mm	Weak
NT5	2.4 mm	0.7 mm	1.6 mm	3.7 mm	2.1 mm	Weak
NT6	3.8mm	0.8 mm	2.2 mm	3.5 mm	2.5 mm	Weak
Kontrol (+)	24 mm	25 mm	26.8 mm	24.2 mm	25 mm	Very Strong
Kontrol (-)	-	-	-	-	-	-

- Values are taken from the average measurement results of 4 repetitions
- Positive control (+) : chloramphenicol
- Negative control (-) : distilled water

Based from the results of the research, isolates from NT1 produced the largest inhibition zone value with an average value of 12.6 mm on *Staphylococcu s aureus* test bacteria and *on Escherichia coli* test bacteria with an average value of 8.95 mm. Based on the measurement data of the inhibition zone of LAB isolates from Tanjungbalai Asahan shrimp paste, it is known that the strength of antibacterial power produced by one isolate, NT1 is categorized as strong on *Staphylococcus aureus* test bacteria and categorized as moderate on *Escherichia coli* test bacteria. While the other five isolates are categorized as weak . Based on the results of measuring the inhibition zone on the test bacteria, it was found that the largest clear zone size was formed on the isolate coded NT1. From tables 4.3.2 and 4.3.3, it is also known that the size of the inhibition zone formed in *Escherichia coli* test bacteria is smaller than the size of the inhibition zone formed in *Staphylococcus aureus*

test bacteria with an average clear zone size of 8.95 mm in *Escherichia coli* while in *Staphylococcus aureus* the average inhibition zone size is 12.6 mm.

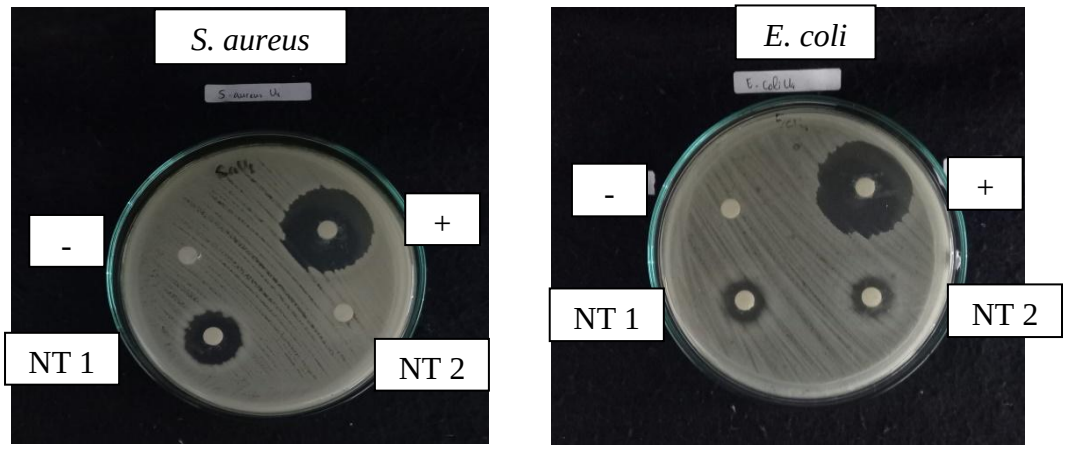

Figure 1. Inhibition zone of lactic acid bacteria from Tanjungbalai Asahan Shrimp Paste, NT1 is categorized as strong on *Staphylococcus aureus* test bacteria and categorized as moderate on *Escherichia coli* test bacteria.

There are several compounds produced by lactic acid bacteria that have anti-bacterial potential, namely bacteriocins, lactic acid, hydrogen peroxide, and carbon dioxide. The strength of secondary metabolic compounds from LAB has different potential depending on the type of sample [12]. Different inhibitory activity is influenced by the type of cell wall that is inhibited, causing differences in the size of the inhibitory area formed in each bacteria.

Based on the results of the pathogenic antibacterial activity test, it can be seen that the 6 LAB isolates from Tanjungbalai Asahan shrimp paste have potential as probiotics, because they fulfill one of the microbial requirements as probiotics, namely having antibacterial activity which can be known if a clear zone or inhibition zone is formed. Probiotics are defined as live mycoorganisms that, if consumed in sufficient quantities, will provide many health benefits. The benefits of probiotics include improving the quality of microflora that has many benefits in the digestive tract, increasing the immune system, reducing ammonia production activity, neutralizing entrotoxins and increasing enzyme work in the digestive system.

Antibacterial activity of pathogens can also be used as an indicator of the quality of shrimp paste from Tanjungbalai Asahan. The higher the inhibition produced by LAB from terasi origin will show the better quality of terasi obtained, because LAB is able to suppress the growth rate of pathogenic bacteria. Based on the results of pathogenic antibacterial activity tests that have been carried out, the six isolates from Tanjungbalai Asahan shrimp paste are able to inhibit pathogenic bacteria characterized by the presence of inhibition zones or clear zones formed. This shows that the quality of Tanjungbalai Asahan shrimp paste is quite good because it contains LAB isolates that have pathogenic antibacterial activity although the inhibition strength produced is still categorized as weak and only one isolate is categorized as strong. In addition, the utilization of lactic acid bacteria isolates can be used as an additional preservative starter that is useful for extending the shelf life of fermented preparations. Product durability is influenced by the activity of inhibition on the growth of spoilage bacteria and pathogenic bacteria due to competition for nutrients and because there are properties of bioactive compounds such as lactic acid,diacetyl and bacteriocins [13].

Table 3.6 Anova Test of Pathogenic Antibacterial Activity (*Staphylococcus aureus*)

Isolate	Inhibition Zone (mm)	p=value
Negative Control (-)	000±000^{a}	0.000
Positive control (+)	23.800±1.4765^{d}	
NT1	12.675±2.0435^{c}	
NT2	2.325±1.5735^{b}	
NT3	0.725±0.2062^{ab}	

NT4	1.075±0.8846[ab]
NT5	0.925±0.0957[ab]
NT6	0.675±0.1258[ab]

Table 3.7 Anova Test of Antibacterial Activity of Pathogens (*Escherichia coli*)

ISOLATE	Inhibition Zone (mm)	p=value
Negative Control (-)	000±000[a]	0.000
Positive Control (+)	25.000±1.2754[d]	
NT1	8.950±2.0664[c]	
NT2	3.125±2.5290[b]	
NT3	1.600±0.7071[ab]	
NT4	2.150±1.0786[ab]	
NT5	2.100±1.2728[ab]	
NT6	2.575±1.3720[ab]	

Based on the results of Anova test on the observation of inhibition zone of LAB isolate from Tanjungbalai Asahan shrimp paste against pathogenic bacteria *E. coli* and *S. aureus* showed a significant level (p = 0.000) which means that the administration of LAB isolate gives a real effect on both pathogenic bacteria. The six LAB isolates from Tanjungbalai Asahan influenced the pathogenic bacteria characterized by the formation of inhibition zone. This study is in accordance with previous research, namely Munaroh, which states that 6 LAB isolates produced from shrimp paste are able to inhibit the growth of *E. coli* and *S. aureus*, and research conducted by Haitham also states that there are 7 LAB isolates from shrimp paste that produce inhibition zones against *S. aureus* and *E. coli.*

4. Conclusion

There are 6 isolates of Lactic Acid Bacteria from Tanjungbalai Asahan shrimp paste that are declared as potential probiotics based on the acidity resistance test (pH) which obtained positive results and have pathogenic antibacterial activity. Where the six isolates have been identified as belonging to 3 genera that have diverse characteristics, namely Pediococcus, Weisella and Lactobacillus. The six LAB isolates from Tanjungbalai Asahan shrimp paste had antibacterial activity against Escherichia coli and Staphylococcus aureus test bacteria with inhibition strength categorized as strong in 1 isolate (NT1) with an average inhibition zone size of 12.6 mm on S. aureus test bacteria and 8.95 mm on E. coli test bacteria while the other five isolates had inhibition strength categorized as weak with an average inhibition zone size of 0.7 mm - 2.5 mm

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