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Identification and antimicrobial activity of lactic acid bacteria from the digestive tract of eels (*Monopterus albus*)

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Abstract. *Ridwan, Retnaningrum E, Ilmi M, Daryono BS. 2019. Identification and antimicrobial activity of lactic acid bacteria from the digestive tract of eels (*Monopterus albus*). Bioteknologi 16: 5-10.* This study aims to identify the species of lactic acid bacteria from the digestive tract of eels (*Monopterus albus*) which have inhibitory activity against pathogenic bacteria. The eel used in this study was obtained from the eel cultivation site in Yogyakarta. The isolation results obtained thirteen isolates, and the isolated bacteria were then phenotypically characterized to select lactic acid bacteria. The results of phenotypic characterization obtained seven isolates that were in accordance with the character of lactic acid bacteria. Seven lactic acid bacterial isolates were then screened to select the three best isolates in inhibiting the growth of pathogenic bacteria (*Aeromonas hydrophila, Staphylococcus aureus, and Vibrio harveyi*). The results showed that seven isolates of lactic acid bacteria had antimicrobial activity which was able to inhibit the growth of pathogenic bacteria. Three of the best isolates were selected to be used for molecular identification and monitoring of antimicrobial activity. The results showed that the three selected isolates produced an antimicrobial extract which was able to inhibit the growth of pathogenic bacteria. Furthermore, the results of the molecular identification showed that the three selected isolates were *Lactococcus lactis* species.

Keywords: 16s rRNA gene, antimicrobial, eel, lactic acid bacteria, *Monopterus albus*

INTRODUCTION

Lactic acid bacteria have various important roles for host health, for example, stimulating the host's immune system (Gatesoupe 2008). In addition, lactic acid bacteria have antimicrobial activity due to the production of lactic acid, acetic acid, bacteriocin, bacteriocin-like compounds and bioactive molecules such as ethanol, formic acid, fatty acids, hydrogen peroxide, diacetyl, reuterin, reutericyclin (Awen et al. 2012). The antimicrobial activity of acid bacteria in inhibiting the growth of pathogenic bacteria attracted the attention of researchers to isolate these bacteria in various sources.

A source that can be used to isolate lactic acid bacteria is the digestive tract of fish. Previous researchers have used the digestive tract of fish to isolate lactic acid bacteria. Lactic acid bacteria isolated from the digestive tract of fish generally have antimicrobial activity which is stable to high temperatures, pH and stable to organic solvents such as chloroform. Loh et al. (2017) reported that *Lactococcus lactis* subsp. lactis CF4MRS isolated from the digestive tract of fish can inhibit bacterial growth of *Escherichia coli*, *Edwardsiella tarda*, *Aeromonas hydrophila*, *Pseudomonas fluorescens*, *Pseudomonas aeruginosa*, *Klebsiella pneumonia* and *Serratia marcescens.*

One group of fish that can be used as a source for isolating lactic acid bacteria is eel (*Monopterus albus*). Eels are aquaculture animals that are very tolerant of low dissolved oxygen content and can live in temperature which is close to freezing (Roy 2013). The ability of eel to live (*Monopterus albus*) on low oxygen content and near-freezing temperatures is likely due to the presence of superior lactic acid bacteria compared to other aquaculture animals. So, further research regarding the antimicrobial characterization of lactic acid bacteria from the eel digestive tract (*Monopterus albus*) is needed. In addition, molecular identification is needed to find out the species of lactic acid bacteria which have the best antimicrobial activity.

MATERIALS AND METHODS

Procedures

Isolation of lactic acid bacteria

Isolation of lactic acid bacteria in this study used eel (*Monopterus albus*) obtained from the Pingit Market, Yogyakarta, Indonesia. Three eels were taken to the Microbiology Laboratory, Faculty of Biology, University Gadjah Mada, Yogyakarta. The eels were then differentiated and the intestines were taken and then mashed using mortar. After that, isolation was carried out using a dilution technique with the pour plate method. The sample was diluted using sterile distilled water, and then a 400 µL 10^{-2} to 10^{-6} diluent was put into a petri dish and then added to MRSA liquid. Petri dishes that have been added with the sample and MRSA media were then incubated at room temperature for 2 x 24 hours. The

growing bacterial colonies were selected based on the formation of clear zones around the colonies for purification. The purification results were propagated on the sloping MRSA media and stored in the refrigerator as stock for further research (Bajpai et al. 2016).

Screening of inhibitory activity of lactic acid bacteria

Screening of lactic acid bacteria used the Well diffusion method. The steps were, first, making a 5 mm well in the NA media (agar nutrient) in a petri dish which has previously been tested by bacterial culture (Aeromonas hydrophila, Vibrio harveyi, Staphylococcus aureus) using the pour plate method, next, adding of 50 µL of lactic acid bacteria culture into the well, then, incubating the well for 24-48 hours at room temperature. The clear zone formed after incubation was measured as the activity of inhibiting lactic acid bacteria (Edalatian et al. 2015).

Antimicrobial production

Lactic acid bacteria culture was grown in 20 mL MRSB media, then incubated in the shaker incubator for 24 hours at 37oC. After 24 hours incubation, the culture was centrifuged at 10,000 rpm at 10ºC for 15 minutes. Then the supernatant was taken as a crude antimicrobial extract and stored in the refrigerator (Hyronimus et al. 1998, Bizani and Brandelli 2002).

Cell-Free Supernatant (CFS) test

Test of CFC's activity used the Well diffusion method. The steps taken were, first, making a 5 mm well on NA media (agar nutrient) in a petri dish which has previously been added to test bacteria (*Aeromonas hydrophila, Vibrio harveyi, Staphylococcus aureus*) using the pour plate method, next, adding 50 µL of antimicrobial crude extract which was filtered using a bacterial filter measuring 0.22 µL pore, then, incubating the well for 24-48 hours at room temperature. The clear zone formed was measured and calculated using the formula:

$$Inhibition\ Score\ (IS) = \frac{\text{Ø clear zone of inhibition (mm)(mm)}}{\text{Ø size of well (mm)}}$$

Antimicrobial inhibitory activity of CFC (Cell-Free Supernatant) is said to be low ($1 < IS < 3$), moderate ($3 \leq IS < 5$), strong ($5 \leq IS < 7$), or very strong ($7 \leq IS < 9$) (Tremonte et al. 2017).

Molecular identification

Bacterial DNA extraction

DNA extraction was carried out by following the procedure contained in the Geneaid Presto™ Mini gDNA Bacteria Kit. The step was to prepare bacterial culture on MRSB media for 48 hours. Bacterial culture of 1.5 mL was, then, transferred to the microcentrifugation tube, and centrifuged for 1 minute at a speed of 14-16,000 x g and then the supernatant was removed. 200 µL gram (+) buffer was inserted into the centrifuge tube and lysozyme (4 mg/mL) was added and then it was vortexed until lysozyme was completely dissolved. A gram (+) of buffer solution that has been added to lysozyme was put into the sample then the pellet is resuspended using a vortex or pipette. The samples were then incubated at 37 ° C for 20 minutes, and it was reversed several times during incubation. Next, 20 µL proteinase K was added and vortexed until homogeneous. It was incubated at 60ºC for 10 minutes, it was reversed every 3 minutes during incubation. The next stage was the sample was added with 200 µL GB buffer then was vortexed, and after that, it was incubated for 10 minutes at 70ºC. The next was the sample was added with 200 µL ethanol absolute and then was vortexed for 10 seconds and then put into GD column and centrifuged at a speed of 14-16,000 g for 2 minutes. The previously used tube was replaced with a new one. After that, 400 µL of W1 buffer was added and it was centrifuged at a speed of 14-16,000 g for 30 seconds. Then 600 µL of W1 buffer was added and centrifuged at a speed of 14-16000 g for 30 seconds, and then GD column was transferred into a sterile Eppendorf tube and added with 100 µL of pre-heated elution and after that, it was centrifuged at a speed of 14-16000 g for 3 minutes. The supernatant which is still in the Eppendorf cane is DNA extract (Geneaid Biotech Ltd., 2017).

Amplification of the 16S rRNA gene Lactic Acid Bacteria

The amplification reaction was carried out in a PCR tube. The amplification used TaKara Eq Taq as much as 0.25 µL, 10 x Ex Taq Buffer (containing Mg2 + 2.5 mM) as much as 5 µL, dNTP mixture (2.5 mM) as much as 4 µL, primer 27f (5 '-AGAGTTTAGTCCTGGCT CAG-3') and 1492r (5'-GGTTACCTTGTTA CGACTT-3 ') with 0.5 µL each, 1 µg of the genome extract and 10 µL of distilled water added. Amplification was carried out to obtain a single band, and the amplification process was initiated with pre-denaturation at 96ºC for 4 minutes, followed by 30 cycles at 94ºC for 1 minute, and annealing at 51.5ºC for 1.30 minutes. After extension at 68ºC for 8 minutes, PCR was completed by elongation for 10 minutes at 68 °C. PCR products were then taken and stored at 4 ° C and examined using 1% agarose (Lawalata et al. 2011).

Agarose electrophoresis

1% agarose was heated to boiling with TAE buffer solvent (tris acetate). After it was cooled, and poured it into the electrophoresis tray and left to be solidified, then the comb was lifted. Furthermore, into the electrophoresis tool, 1x TAE buffer was poured. The tray containing the solid agarose was then placed on electrophoresis until it was submerged by a buffer. The loading dye was prepared on top of the parafilm then was mixed with 4 µL PCR products and put into agarose well using a micropipette, in addition, a 3 µL marker was prepared. The electrophoresis device was conducted at a voltage of 100 volts for 30 minutes. Agarose results from electrophoresis were immersed in an ethidium bromide dye solution for 10 minutes, then they were visualized in the transilluminator, The presence of DNA bands with a size of ± 1500 bp indicated that the 16S rRNA gene has been amplified, then the results were documented using a camera.

Data analysis

The results of 16S rRNA gene sequencing were analyzed on the website of www.ncbi.nlm.nih.gov. The BLAST program was used to determine the similarity of nucleotide sequences so that identification can be done based on the similarity of nucleotide sequences. Then, Phylogeny Tree Construction was carried out to determine the level of closeness between species using the application of MEGAX (Kumar et al. 2018).

RESULTS AND DISCUSSION

Isolation of lactic acid bacteria

Thirteen isolates from isolates in this study were phenotypically characterized. Phenotypic characterization being carried out was colony morphology, cell morphology, catalase test and gram staining properties. The results of the phenotypic characteristics of the thirteen bacterial isolates are listed in Table 1.

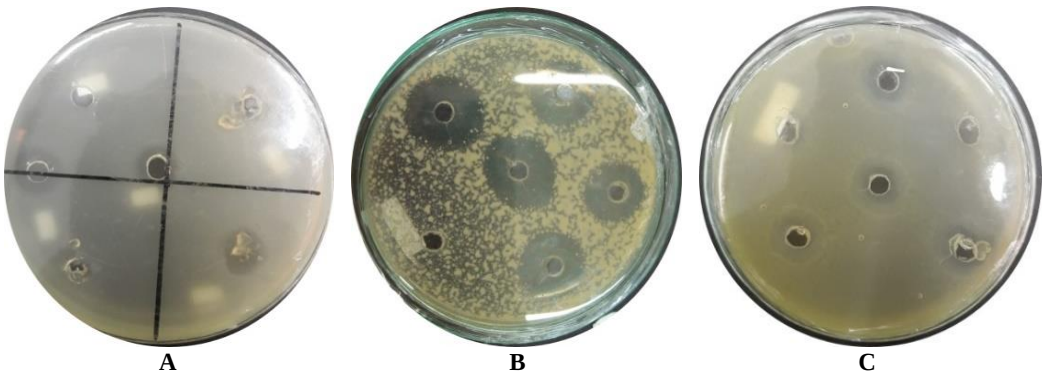
The results of phenotypic characterization listed in Table 1 show that there are seven bacterial isolates that are suitable for the characterization of lactic acid bacteria. These seven isolates were then screened to find three isolates that had the best antimicrobial activity in inhibiting the growth of test bacteria (*Aeromonas hydrophila*, *Staphylococcus aureus*, and *Vibrio harveyi*).

Screening of inhibitory activity of lactic acid bacteria

Screening the ability of lactic acid bacteria is carried out on seven bacterial isolates. This screening aims to select lactic acid bacteria which have the best activity in inhibiting the growth of pathogenic bacteria (*Aeromonas hydrophila, Staphylococcus aureus* and *Vibrio harveyi*). The inhibition ability is best seen based on the diameter of the clear zone formed around the well (Figure 1). The results of screening for inhibitory activity of lactic acid bacteria are listed in Table 2.

Table 1. Characteristics of lactic acid bacteria strain isolated from eels (*Monopterus albus*)

Character	Bacterial Isolates												
	B1A	**B1B**	**B1C**	**B1D**	**B1E**	**B1F**	**B1G**	**B1H**	**B1I**	**B1J**	**B1K**	**B1L**	**B1M**
Cell shape	Cocci	Cocci	Cocci	Cocci	Cocci	Cocci	Cocci	Cocci	Cocci	Cocci	Cocci	Cocci	Cocci
Gram strain	+	+	+	+	+	+	+	-	-	+	-	-	-
Catalase	-	-	-	-	-	-	-	+	+	+	+	+	+
Color													
White	-	-	+	+	-	+	+	+	+	+	+	+	+
Cream	+	+	-	-	+	-	-	-	-	-	-	-	-
Size													
Small	+	+	+	+	-	+	-	+	+	-	-	+	+
Pintpoint	-	-	-	-	+	-	+	-	-	+	+	-	-
Elevation													
Convecs	+	-	+	+	+	+	+	+	+	+	+	+	+
Flat	-	+	-	-	-	-	-	-	-	-	-	-	-



A **B** **C**

Figure 1. Zone of inhibition of lactic acid bacteria at the screening stage i.e.: A. Inhibition zone of *Aeromonas hydrophila*, B. Inhibition zone of *Vibrio harveyi*, C. Zone of inhibition of *Staphylococcus aureus*

Table 2. Screening results of seven isolates of lactic acid bacteria isolated from the eel digestive tract (*Monopterus albus*)

Strain code	Ø Well (mm)	Ø Mean inhibitory zone (mm)		
		Vibrio harveyi	*Aeromonas hydrophila*	*Staphylococcus aureus*
B1A	5	23.33 ± 1.15^{ab}	11.67 ± 0.58^{a}	16.67 ± 0.58^{a}
B1B	5	21.00 ± 1.00^{cd}	11.33 ± 1.15^{a}	14.67 ± 1.15^{ab}
B1C	5	21.00 ± 3.46^{cd}	12.33 ± 2.31^{a}	9.00 ± 1.73^{c}
B1D	5	25.33 ± 2.89^{a}	12.33 ± 2.52^{a}	13.00 ± 2.65^{b}
B1E	5	22.33 ± 3.21^{bc}	12.00 ± 3.00^{a}	11.33 ± 1.15^{bc}
B1F	5	21.00 ± 1.73^{cd}	11.33 ± 1.15^{a}	11.67 ± 2.08^{bc}
B1G	5	18.67 ± 0.58^{d}	11.67 ± 0.58^{a}	13.67 ± 4.04^{ab}
K-	5	-	-	-

Note: Ø = Diameter. Different letters in the same column show the level of real difference - Duncan test α <0.05 (mean ± std)

Table 2 shows that antimicrobial extract of the three isolates had inhibitory activity against the test bacteria (*Aeromonas hydrophila*, *Staphylococcus aureus* and *Vibrio harvei*). The inhibition activities of seven isolates of lactic acid bacteria were then analyzed statistically using SPSS 17.0 software. The results of statistical analysis showed that the inhibitory activity of lactic acid bacteria had a significant effect on inhibiting the growth of pathogenic bacteria. Ringo (2005) states that lactic acid bacteria isolated from aquatic animals have the ability to inhibit the growth of pathogenic bacteria such as *Aeromonas hydrophila* and *Vibrio harveyi*. Buntin et al. (2008) reported that lactic acid bacteria isolated from the digestive tract of fish were able to inhibit the growth of *Staphylococcus aureus* bacteria. Based on the results of the screening, three isolates were selected for further testing. Selected isolates in this study were B1A, B1D and B1E.

Cell-Free Supernatant (CFS) test

The CFC test was conducted to determine the activity of antimicrobial crude extract in inhibiting the growth of test bacteria. The antimicrobial crude extract was obtained from 24-hour-old culture of lactic acid bacteria. Antimicrobial extracts were filtered using a bacterial filter measuring 0.22 µL pore to separate cells from supernatants. The crude extract filtered was tested for its ability to inhibit the growth of test bacteria. The results of the antimicrobial crude extract activity test are listed in Table 3.

Based on Table 3, the ability of antimicrobial extract from the three bacterial isolates is known now. The results showed that the antimicrobial extract of the three isolates had inhibitory activity against the test bacteria. The three isolates had an Inhibitor Score (IS) <3 or a weak category against *Aeromonas hydrophila* and *Vibrio harveyi*. Furthermore, isolates B1A and B1D have Inhibitor Score (IS) <3 or weak categories while isolates B1E have Inhibitor Score (IS) > 3 or moderate categories against *Staphylococcus aureus* bacteria.

Molecular identification

Molecular identification aims to determine the isolate species of lactic acid bacteria. Molecular identification used the 16S rRNA gene. Amplification of the 16S rRNA gene used 27F primers (5'-AGAGTTTA GTCCTGGCTCAG-3 ') and 1492R (5'-GGTT ACCTTGTTACGACTT-3'). The amplification results are listed in Figure 2.

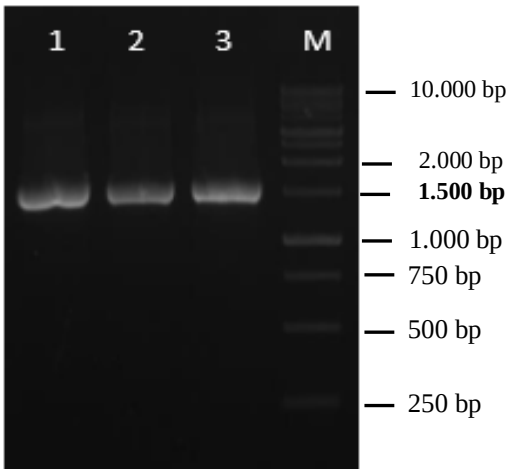


Figure 2. Results of amplification of the 16S rRNA gene

Table 3. Test results for antimicrobial crude extract activity

Test bacteria	Strain code	Ø Well (mm)	Ø Inhibition zona (mm)			Mean Ø Inhibition zona (mm)	*Inhibition Score* (IS)
			U1	U2	U3		
Vibrio harveyi	B1A	5	13	15	14	14.00 ± 1.00	2.80^{a}
	B1D	5	14	13	13	13.33 ± 0.58	2.67^{a}
	B1E	5	15	15	14	14.67 ± 0.58	2.93^{a}
	K-	5	-	-	-	-	-
Aeromonas hydrophila	B1A	5	13	14	14	13.67 ± 0.58	2.73^{a}
	B1D	5	14	15	14	14.33 ± 0.58	2.86^{a}
	B1E	5	14	15	15	14.67 ± 0.58	2.93^{a}
	K-	5	-	-	-	-	-
Staphylococcus aureus	B1A	5	15	15	14	14.67 ± 0.58	2.93^{a}
	B1D	5	15	14	15	14.33 ± 0.58	2.86^{a}
	B1E	5	14	16	16	15.78 ± 1.15	3.16^{b}
	K-	5	-	-	-	-	-

Note: Ø = diameter. ^{a} = weak, ^{b} = moderate, ^{c} = strong

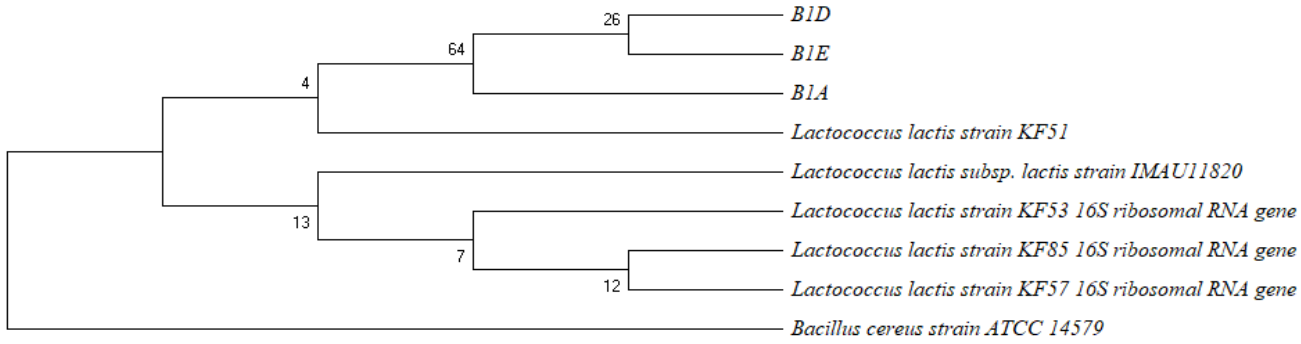


Figure 3. Tree of phylogeny isolates of lactic acid bacteria from eel (*Monopterus albus*) (bootstrap = 1000)

The results of 16S rRNA gene amplification were then sequenced using the same primer as the amplification process, 27F and 1492R primers. The sequencing results were then analyzed using BLAST on the site of www.ncbi.nlm.nih.gov with a database of ribosomal 16S rRNA genes. The BLAST analysis results showed that the three isolates had 99% similarity with *Lactococcus lactis* species. Then the phylogenetic tree construction was carried out to determine the evolutionary relationship between bacterial species from isolation and BLAST results. The phylogenetic tree was reconstructed using MEGAX software. The results of the phylogenetic tree reconstruction are shown in Figure 3.

The phylogeny tree in Figure 3 shows that isolates B1A, B1D, and B1E isolated from the eel digestive tract were in one cluster with *Lactococcus lactis*. The results showed that the isolates of lactic acid bacteria isolated from the eel digestive tract (*Monopterus albus*) are a species of *Lactococcus lactis.* Marrifield et al. (2014) reported that lactic acid bacteria include indigenous bacteria in the digestive tract of fish. Lactic acid bacteria that occupy the digestive tract of fish include *Lactococcus lactis*, *Streptococcus* spp., *Weissella cibaria*, and *Lactobacillus* spp. Kato et al. (2016) reported that Lactococcus spp. from the digestive tract of fish have antimicrobial activity and can inhibit the growth of *Staphylococcus aureus* (inhibitory zone 6 ± 0.17), *Escherichia coli* (inhibition zone 7 ± 0.10) and *Proteus* spp. (3.5 ± 0.10).

Discussion

Lactic acid bacteria have many important roles, for example, it can stimulate the host's immune system (Verschuere et al. 2000), and it can also produce antimicrobial compounds that can inhibit the growth of harmful pathogenic bacteria (Muthukumar and Kandepan 2015). Previous studies by Balcazar et al. (2008) reported that lactic acid bacteria isolated from the digestive tract of fish were able to inhibit the growth of pathogenic bacteria such as *Aeromonas hydrophila, Aeromonas salmonicida, Yersinia ruckeri dan Vibrio anguillarum.*

This study shows that isolated lactic acid bacteria from the eel digestive tract could potentially inhibit the growth of pathogenic bacteria. Lactic acid bacteria that have the potential to inhibit the growth of pathogenic bacteria are then identified molecularly. The purpose of this study was to find lactic acid bacteria that can inhibit the growth of *Vibrio harveyi*, *Staphylococcus aureus*, and *Aeromonas hydrophila*. *Staphylococcus aureus* is one of the food pathogenic bacteria found throughout the world. Then *Vibrio harveyi* is one of the causes of gastroenteric disease and tissue invasion, especially in hosts who experience a decline in the immune system (Auerbach et al. 2016). And then *Aeromonas hydrophila* is one of the causes of infectious diseases in fish (Percival and Williams 2014). It is needed to conduct the effort to look for bacteria that have the potential to inhibit the growth of the three pathogenic bacteria. This study found that lactic acid bacteria were able to inhibit the growth of *Vibrio harveyi*, *Staphylococcus aureus*, and *Aeromonas hydrophila*. These lactic acid bacteria are included in the species *Lactococcus lactis*.

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