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Lactic Acid Bacteria from Mangrove Sediment Produce Bacteriocins Active Against Gram-Positive and Negative Bacteria

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Abstract. *Mangrove is a unique ecosystem. Only a few studies have explored the presence of lactic acid bacteria and their roles in mangrove ecosystems. From mangrove sediments at Logending Beach in Jawa Tengah (Indonesia), the Lactic Acid Bacteria (LAB) isolates LG-50, LG-107, and LG-114 were discovered. They produce bacteriocins. This study aimed to determine the characteristics of LAB isolates, antimicrobial activity, and physicochemical properties of bacteriocins. LAB isolates were characterized by morphology, physiology, and biochemistry. The production of bacteriocin was performed by salting-out method, followed by testing its antimicrobial activity against pathogenic bacteria. Isolates LG-50, LG-107, and LG-114 are thought to be in the Lactobacillus group. The crude bacteriocin can inhibit the growth of Gram-positive and negative bacteria. The average inhibition zones against Escherichia coli and Staphylococcus aureus were 16.67 mm and 22.17 mm, respectively. The crude bacteriocin tested positive for ninhydrin. It confirmed the crude bacteriocin was a protein and sensitive to the proteolytic enzyme. SDS-PAGE analysis presented the molecular weight of crude bacteriocin was 38 kDa. This present study supports the potential use of bacteriocin in the pharmaceutical and food industries.*

Keywords: *antimicrobe, bacteriocin, broad-spectrum, Lactobacillus, mangrove, SDS-PAGE*

Citation

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INTRODUCTION

Mangrove is a unique ecosystem because it has strong interactions with the ocean, estuaries, rivers, and inland waters. The mangrove environment protects the shoreline and helps to prevent erosion (Wickramasinghe et al., 2009). It accumulates sediments, contaminants, and nutrients. Due to this condition, mangrove ecosystems have high biodiversity, including flora, fauna, and microorganisms (Mendes & Tsai, 2014; Martuti et al., 2018). Haldar & Nazareth (2018) reported the diversity of Gammaproteobacteria, Alphaproteobacteria, Deltaproteobacteria, and Bacilli in Mandovi and Zuari mangrove sediments.

Microorganisms play an active role in the ecological cycles that provide resources to animals and plants in mangrove environments. Organic matter is commonly found in soil sediments. They nevertheless lack phosphorus and nitrogen, necessitating the par-

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ticipation of microbes in nitrogen fixation, hydrocarbon decomposition, phosphate solubilization, sulfate reduction, photosynthetic assimilation, and enzyme production (Sahoo & Dhal, 2009; Ismail et al., 2017). Bacteria from mangrove ecosystems may be biotechnologically valuable in addition to their ecological roles. Only a few studies have explored the presence of lactic acid bacteria and their roles in mangrove ecosystems. For example, Thiruneelakandan et al. (2014) succeeded in isolating lactic acid bacteria that have the potential to produce antimicrobial compounds.

Lactic acid bacteria (LAB) produce bacteriocins, organic acids, hydrogen peroxide, and lactic acid. Bacteriocins are reported to have an antimicrobial effect against various bacteria, both pathogenic and non-pathogenic bacteria (Daba & Elkhateeb, 2020; Da-Costa et al., 2019; Suardana et al., 2017). Therefore, the use of bacteriocins can be an alternative to handling microbial resistance to antibiotic compounds.

Previous research has succeeded in discovering several isolates of "lactic acid bacteria from mangrove sediments at Logending Beach in Jawa Tengah (Kusharyati et al., 2021). This present study aimed to determine the characteristics of LAB isolates from mangrove sediment, bacteriocin activity as an antimicrobial against *Staphylococcus aureus* and *Escherichia coli*, and the physicochemical characteristics of bacteriocins.

MATERIALS AND METHODS

This research was conducted at the Faculty of Biology and Research Laboratory of Universitas Jenderal Soedirman Purwokerto and The Institute of Tropical Diseases of Universitas Airlangga Surabaya from July – November 2022. This present study used several reagents and bacterial growth media, including de Man Rugose Sharpe (MRS) agar, MRS broth, Mueller Hinton Agar (MHA), Sulfide Indole Motility (SIM) agar, Nutrient agar, Nutrient broth, 6 mm paper disc, physiological NaCl, crystals violet, Lugol's iodine, safranin, H_2O_2 3%, malachite green, tetramethyl-p-phenylenediamine dihydrochloride, $CaCO_3$, NaOH, HCl, ammonium sulfate, tetracycline, ninhydrin solution, resolving gel, stalking gel, loading buffer, Coomassie brilliant blue, ThermoFisher Protein Standard 3-198 kDa, and phosphate buffer 0.1 M.

LAB Isolate Subculture

LAB isolates were isolated from mangrove sediments at Logending Beach in Jawa Tengah, Indonesia. LAB isolates were grown on deMann Ragosa Sharpe Agar medium (MRSA, Oxoid) supplemented by 1% CaCO3. The medium was incubated at 37 °C for 48 hours. A single colony was inoculated on MRSB liquid media (Oxoid) for further analysis.

Morphological Characterization

Macromorphological observation: the observation of colonies was carried out by observing the shape of the colony, the color, the edge, the surface, and the elevation of the single colony.

Micromorphological observation, 1) Gram staining: the staining result was observed under a light microscope. Gram-positive bacteria were presented in purple. Cell shape and cell configurations were regarded as cellular morphological parameters throughout the observation; 2) Endospore staining: LABs do not produce endospores. Vegetative cells were presented in red; 3) Motility test: LAB isolates were inoculated on Sulfide Indole Motility Agar medium (SIMA, Oxoid) and then incubated at 37 °C for 48 hours. The result was declared non-motile when the only

visible spread was at the puncture site of the inoculation (Cappuccino & Sherman, 2014).

Physiological Characterization

pH Test: LAB isolates were grown on an MRSB medium with various pH values (pH 3, 5, 6, and 9), and then incubated at 37 °C for 24 hours. The turbidity of the medium revealed the colony's growth. Temperature test: LAB isolates were grown on an MRSB medium and then incubated at various temperatures for 24 hours (5 °C, 37 °C, and 45 °C). The turbidity of the medium revealed the colony's growth. Salinity testing: LAB isolates were grown on MRSB medium and supplemented with various concentrations of NaCl (3%, 5%, 6.5%, and 10%), after which the medium was incubated for 7 days at 37 °C. The turbidity of the medium revealed the colony's growth. Oxygen requirement test: a total of 0.1 ml of LAB liquid culture was grown on an MRSB medium and then incubated for 48 hours at 37 °C. The result was declared facultatively anaerobic when bacteria grew over the entire surface of the liquid medium (Cappuccino & Sherman, 2014).

Biochemical Characterization

Catalase test: LAB isolate was smeared on the glass object and then 3 drops of a 3% H2O2 solution were added. The catalase tested positive when air bubbles formed. Oxidase test: LAB isolate was smeared on glass objects and then covered with grey paper. A total of 3 drops of the tetramethyl-p-phenylenediamine dihydrochloride reagent were added to the smeared cells. The oxidase tested positive when the smeared cells were turning blue-black. Fermentation type: LAB isolate was grown on Nutrient Broth (Oxoid) medium containing Durham tubes. The medium was incubated for 48 hours at 37 °C. Heterofermentative bacteria produce gas, while homofermentative bacteria do not produce gas (Cappuccino & Sherman, 2014).

Production and Extraction of Bacteriocins

LAB isolates were inoculated on 10 mL of Nutrient Broth medium and then incubated for 18 hours at 37 °C. A total of 2.5 mL of LAB liquid culture (aged 18 hours, ~10^8 CFU/mL) was inoculated on 250 mL of Nutrient Broth medium and then incubated for 18 hours at 37°C. The liquid culture was centrifuged at 5,480 x g for 15 minutes at 4 °C. The resulting cell-free supernatant (CFS) was separated using the salting-out method by adding ammonium sulfate (Kusharyati et al., 2021). A solution of ammonium sulfate (60%) was slowly added until homogeneous. The mixture was centrifuged at 5,480 x g for 30 min at 4 °C. The precipitate was dissolved in a 0.1 M phosphate buffer at pH 5 and then stored as crude bacteriocin.

Influence of Proteolytic Enzyme

Papain was used to analyze the sensitivity of crude bacteriocin against proteolytic enzymes (Kusharyati et al., 2021). A total of 200 µl of crude bacteriocin was added, along with 20 µl of papain enzyme (the pH of the mixture was adjusted to 7). The mixture was incubated for 2 hours at 37 °C. A total of 20 µl of the mixture was dripped on disc paper (6 mm in diameter, Oxoid) and placed on Mueller Hinton Agar (Oxoid) medium overgrown with indicator bacteria (~10^8 CFU/ml of *Staphylococcus aureus* and *Escherichia coli*). The medium was incubated for 24 hours at 37 °C. The active crude bacteriocin produces an inhibitory zone around the disc paper.

Antimicrobial Assay

The antimicrobial assay was performed using the disc diffusion method (Hudzicki, 2009). A total of 50 µl of the test compound

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(tetracycline, aquadest, crude bacteriocin + papain, and crude bacteriocin) was dripped on disc paper (6 mm in diameter, Oxoid) and placed on Mueller Hinton Agar media overgrown with test bacteria (~10^8 CFU/ml of *Escherichia coli* and *Staphylococcus aureus*). The medium was incubated for 24 hours at 37 °C. Compounds that have antimicrobial activity produce an inhibitory zone around the disc paper. The inhibitory zone was measured by the following formula:

$$D\ (mm) = ((D1+D2))/2$$

Note:
D : total diameter of the inhibitory zone
D1: vertical diameter of the inhibitory zone
D2: horizontal diameter of the inhibitory zone

Ninhydrin Test

A 0.1% solution of ninhydrin was dripped onto 1 ml of crude bacteriocin and then heated to a boil. The positive reaction was characterized by a change in color to purple (Suardana et al., 2017).

Characterization of Bacteriocin

The SDS-PAGE method was used to determine the molecular weight of crude bacteriocin (Suardana et al., 2017). The discontinuous gel consists of 4% stacking gel and 12.5% separating gel. The gel was put into the mold, and then we waited until it was solid. A total of 10 µl of crude bacteriocin was mixed with 30 µl of loading buffer solution and then heated at 90 °C for 5 minutes. Standard proteins measuring 3-198 kDa were used as markers (Thermo Fisher). The running buffer was inserted into the gel. A total of 5 µl of crude bacteriocin was introduced into the well. The process took an hour. The gel was removed and placed in a container containing Coomassie Brilliant Blue, then stored at 37 °C for 24 hours. The determination of the molecular weight of bacteriocin was compared with marker protein bands.

RESULTS AND DISCUSSION

The LAB isolates from mangrove sediment at Logending Beach, Kebumen in Jawa Tengah are suspected to be the Lactobacillus group, i.e., isolates Logending 50 (LG-50), Logending 70 (LG-70), and Logending 114 (LG-114). The crude bacteriocins inhibit the growth of *Staphylococcus aureus* and *Escherichia coli* in vitro.

Foodborne bacteria are becoming more resistant to a variety of widely viable antibiotics. Many studies have shown that LAB can inhibit the growth of food pathogens both in vitro and in vivo (Manzoor et al., 2016). Among LABs, Lactobacilli and Cocci have been the most often employed LABs in the food industries.

Thiruneelakandan et al. (2014) succeeded in isolating Lactobacillus from mangrove ecosystems. These isolates are reported to produce bacteriocin against bacteria that cause seafood spoilage. Similarly, Sukmawati et al. (2022) isolated *Bacillus australimaris* from mangrove ecosystems that can be used as biological controllers. It can inhibit the growth of the rice blast pathogenic fungus (*Magnaporthe oryzae*). This lactic acid bacterium is found in fermented foods and can be utilized as a probiotic (Chen et al., 2019).

LAB produces a variety of metabolites in addition to bacteriocins, including vitamins, organic acids, short-chain fatty acids, amines, and exopolysaccharides. Bacteriocin is generally recognized as safe. It continues to interest many researchers because it has beneficial use in the food and pharmaceutical industries.

Characterization of LAB isolates

A single colony of LAB isolates has the characteristics of a shiny smooth colony surface, circular shape, milky white color, convex elevation, convex edge, small size, opaqueness, and no pigment (Figure 1). Based on Bergey's Manual of Determinative Bacteriology, this characteristic has similarities with the group of *Lactobacillus*. Micromorphological observation confirms that all LAB isolates are Gram-positive, do not form endospores, and are non-motile (Table 1). Lactobacillus is generally non-motile, although some *Lactobacillus* have flagella and exhibit motile properties (Kajikawa et al., 2016).

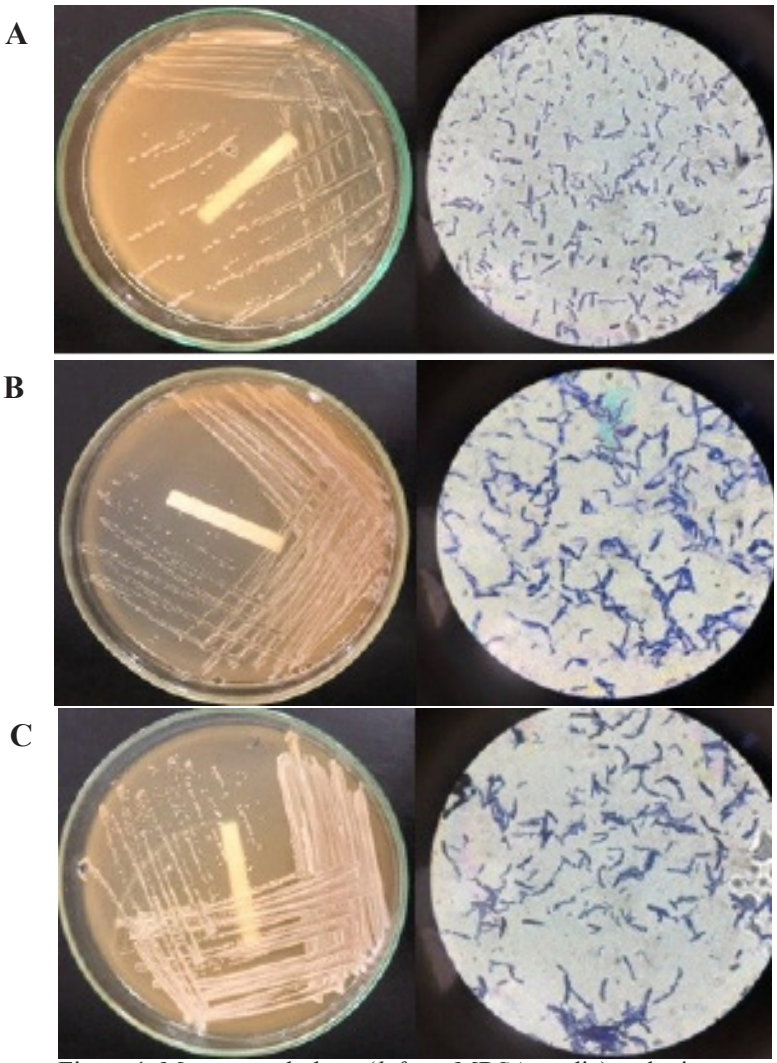

Figure 1. Macromorphology (*left,* on MRSA media) and micromorphology (*right*, 1000x magnification on a light microscope). Note: a) Isolate LG-50, b) LG-107, and c) LG-114.

The growth test results show that all LAB isolates grow optimally at 37 °C and can survive at 45 °C. However, LAB isolates cannot grow at 5 °C (Table 1). Azhara et al. (2022) reported that LAB growth was optimum at 37 °C and began to occur at 10 °C. LAB iso-

lates can grow on MRSB media with pH 5, pH 6.5, and pH 9. However, isolates cannot grow at pH 3. Van-Thuoc et al. (2021) found that Lactobacillus can live at pH 4–10 and has an optimum pH of 6–6.5. LAB isolates can also grow on an MRSB medium containing 3% to 6.5% of NaCl. Similarly, Laily et al. (2013) reported the growth of *Lactobacillus* in the medium containing NaCl in the range of 3–7%. LAB isolates are facultatively anaerobic and homofermentative. Lactic acid bacteria from the *Lactobacillus* group are facultatively anaerobic (Hendarto et al. 2019). The bacteria can live with or without oxygen. Homofermentative LAB converts glucose to produce lactic acid as the main product (Anindita et al., 2022).

The degree of bacterial inhibition was not only related to the production of lactic or acetic acid. "The presence of numerous chemicals, such as organic acids, H2O2, and bacteriocin, may be substantially connected to the antimicrobial capabilities of LABs (Leslie et al., 2021). The application of the proteolytic "papain" enzyme to bacteriocin activity showed that no inhibitory zone was formed around the disc paper against the indicator bacteria. It confirms that the antimicrobial compounds produced by LAB isolates (LG-50, LG-107, and LG-114) are bacteriocins.

Table 1. Phenotypic characterization of LAB isolates

Characters	Isolates		
	LG-50	LG-107	LG-114
Macro-morphology			
Colony surface	Smooth Circular	Smooth Circular	Smooth Circular
Colony form	Glazed	Glazed	Glazed
Colony color	Milky White Convex	Milky White Convex	Milky White Convex
Colony elevation	Flat	Flat	Flat
Colony edge	Small	Small	Small
Colony size	Opaque	Opaque	Opaque
Colony optics	None	None	None
Pigmentation			
Micromorphology			
Grams	Positive	Positive	Positive
Cell shape	Bacil	Bacil	Bacil
Cell arrangement	Strepto	Strepto	Strepto
Endospores	None	None	None
Motility	-	-	-
Bacterial growth			
Temperature 5°C	-	-	-
Temperature 37°C	+	+	+
Temperature 45°C	+	+	+
pH 3	-	-	-
pH 5	+	+	+
pH 6	+	+	+
pH 9	+	+	+
NaCl 3%	+	+	+
NaCl 5%	+	+	+
NaCl 6,5%	+	+	+
NaCl 10%	-	-	-
Oxygen requirements	Facultative anaerobic	Facultative anaerobic	Facultative anaerobic

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Biochemistry			
Catalase	-	-	-
Oxidase	+	+	+
Fermentation type	Homofermentative	Homofermentative	Homofermentative
Genus's identification based on Bergey's Manual of Determinative Bacteriology	Lactobacillus	Lactobacillus	Lactobacillus

Note:
(+): Positive interpretation
(-): Negative interpretation

Bacteriocin Production and its Antimicrobial Activity

Bacteriocin is an active protein that is sensitive to proteolytic enzymes, such as trypsin, pepsin, and papain enzymes. Bacteriocin contains disulfide bonds. The disulfide bonds are damaged by proteolytic enzymes, resulting in the loss of bacteriocin activity (Paryati et al., 2022; Sari et al., 2016). It is characterized by the loss of inhibitory zones.

The indicator pathogenic bacteria were *Staphylococcus aureus* and *Escherichia coli.* These bacteria were selected as representing spoilage bacteria of economic importance in food and raw material handling. The antimicrobial effect of bacteriocin from LAB isolates (LG-50, LG-107, and LG-114) is characterized by the presence of an inhibitory zone around the disc paper (Figure 2). *Lactobacillus plantarum*, according to Sari et al. (2016), can suppress the development of *S. aureus*, *Salmonella typhi*, and *E. coli*.

Table 2 suggests that bacteriocin produced a larger inhibitory zone against *S. aureus* than *E. coli*. Gram-positive bacteria (e.g., *S. aureus*) are generally more sensitive to the bacteriocin of LAB isolates LG-50, LG-107, and LG-114 than Gram-negative bacteria (e.g., *E. coli*) (Figure 3). The average diameter of the inhibitory zone against *S. aureus* is 22.17 mm, while that against *E. coli* is 16.67 mm.

The cell wall of Gram-negative bacteria consists of lipoproteins, lipopolysaccharides, and peptidoglycans. "It causes Gram-negative bacteria more difficult to be penetrated by antibacterial compounds than Gram-positive bacteria (Hamidah et al., 2019). There are few studies on LAB's bacteriocin that is active against Gram-negative bacteria, thus the antagonistic action against Gram-negative bacteria is of special interest. Moreover, antagonistic activity against Gram-negative pathogens would enhance its use in food preservation".

Characterization of Bacteriocin

The characterization of bacteriocins was carried out using a ninhydrin test in order to find out the presence of free amino acids in certain materials. A ninhydrin test of three LAB isolates (LG-50, LG-107, and LG-114) showed the formation of purple color in the solution. The purple color of the solution is caused by the reaction between α-amino acids and ninhydrin. Oxidized ninhydrin compounds cause oxidative decarboxylation of α-amino acids, forming hydrindantin, CO2, NH3, and aldehydes (Lestari et al., 2019).

Protein molecular weight testing, also known as SDS-PAGE, was used to characterize bacteriocins. SDS-PAGE aims to separate protein molecules based on the size and shape of their particles. It can determine their

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molecular weight. LAB isolates LG-50, LG-107, and LG-114 have a molecular weight of 38 kDa (Figure 4). It belongs to class III bacteriocins. Rachmania et al. (2017) concluded that class III bacteriocins are generally large, i.e., more than 30 kDa. The results obtained on SDS-PAGE electrophoresis showed a very thin protein band. Bacteriocins, carbon dioxide, hydrogen peroxide, and diacetyl (2,3-butanedione) are among the low-molecular-weight compounds found in LAB (Leslie et al., 2021).

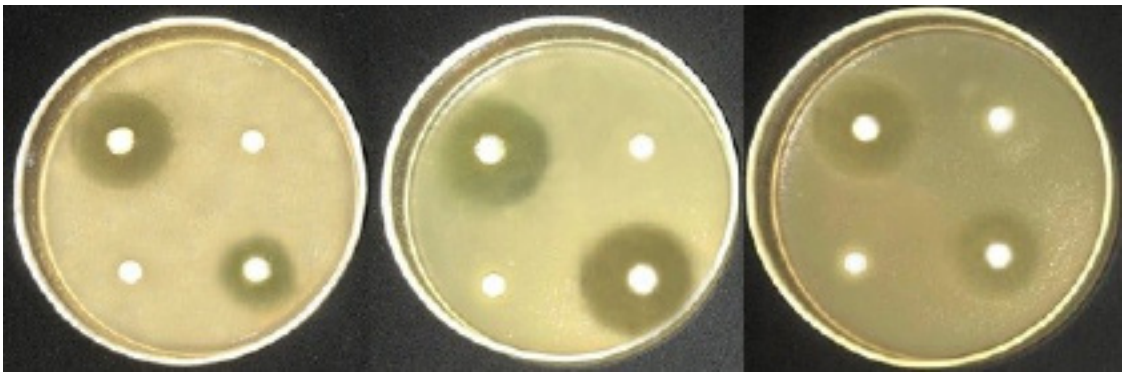


Figure 2. Bacteriocin activity of LAB isolates LG-50 (a), LG-107 (b), and LG-114 (c) against *Staphylococcus aureus*. Note: (1) positive control (tetracycline); (2) negative control (aquadest); (3) bacteriocin + papain enzymes; (4) bacteriocin.

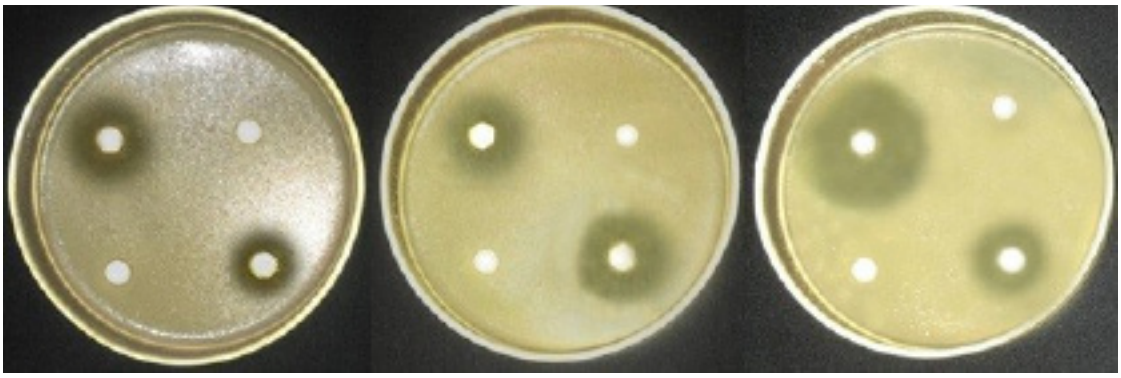


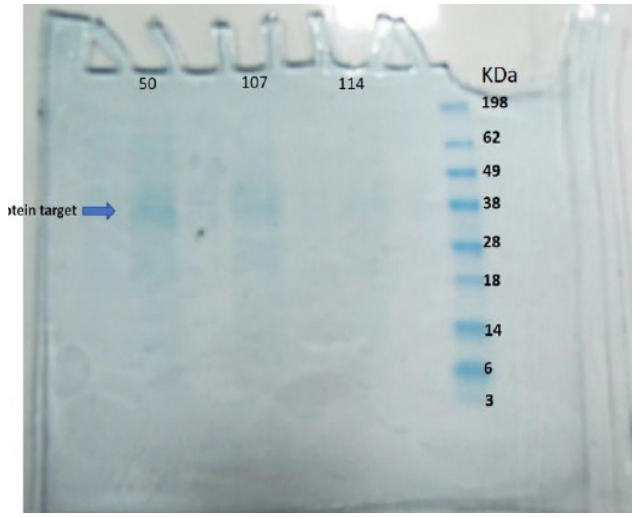
Figure 3. Bacteriocin activity of LAB isolates LG-50 (a), LG-107 (b), and LG-114 (c) against *Escherichia coli*. Note: (1) positive control (tetracycline); (2) negative control (aquadest); (3) bacteriocin + papain enzymes; (4) bacteriocin.

Table 2. Bacteriocin activity of LAB isolates against *Staphylococcus aureus* and *Escherichia coli*

Isolates	**Diameter of the Inhibitory Zone (mm)**	
	Staphylococcus aureus	***Escherichia coli***
LG-50	19	14
LG-107	29	21
LG-114	18.5	15

A thin protein band can be caused by a low concentration of protein due to protein degradation (Wardhani et al., 2021). A decrease in protein solubility can be caused by the salting-out process using ammonium sulfate. The cross-linking of disulfides causes protein solubility to decrease (Rachmania et al., 2017).

Class III bacteriocins have a large molecular mass. For example, a class III bacteriocin, helveticin, is produced by L. helveticus with a size of >37 kDa (Benítez-Chao et al., 2021). Bacteriocin of the *Lactobacillus* group generally has varied molecular weights ranging from 15 to 40 kDa (Islam et al., 2020). Similarly, the bacteriocin of *L. rhamnosus* IN13 was reported to have a molecular weight of 35 kDa (Maulidayanti et al., 2019). The bacteriocin of *L. plantarum* SB7 had a molecular weight of 48 kDa (Mulyawati et al., 2019). Bacteriocin of *Lactobacillus acidophilus* NX2-6 had a molecular weight of 37.1 kDa (Meng et al., 2021). Meanwhile, Iseppi et al. (2019) reported a low molecular weight of bacteriocin produced by several strains of *Lactobacillus*. The bacteriocin of *L. paracasei* and *L. brevis* has a low molecular weight of 3 kDa.



CONCLUSION

LAB isolates LG-50, LG-107, and LG-114 from the mangrove sediments of Logending Beach, Kebumen in Jawa Tengah had similar characteristics to the genus *Lactobacillus*. The crude bacteriocin can inhibit the growth of *Escherichia coli* and *Staphylococcus aureus*. The average inhibitory zones against *E. coli* and *S. aureus* were 16.67 mm and 22.17 mm, respectively." The molecular weight of bacteriocin produced by LAB isolates was 38 kDa.

AUTHOR CONTRIBUTION

Conceptualization: D.F.K., H.P. Methodology: D.F.K., H.P. and F.J.A. Investigation: F.J.A. and A.R. Writing—original draft preparation: D.F.K., F.J.A. and A.R. Writing—review and editing: A.R.

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CONFLICT OF INTEREST

The author declares that there was no conflict of interest in this study.

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