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Unveiling the Antimicrobial of Lactic Acid Bacteria from Gatot Cassava *(Manihot esculenta)* Against *Escherichia coli* and *Salmonella typhi*

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Abstract

Background: *Lactic Acid Bacteria (LAB) as probiotics provide many benefits to the body, including suppressing the growth of pathogens in the digestive tract. Antibacterial compounds from LAB can inhibit the presence of pathogenic bacteria. This study aimed to determine the ability of antibacterial compounds from Gatot LAB to inhibit the growth of Escherichia coli and Salmonella typhi bacteria.* **Methodology:** *LAB was isolated from cassava gatot, and The LAB isolates obtained were then tested for several LAB characteristics and antibacterial activity against E. coli and S. typhi. LAB characterization based on colony morphology, Gram staining, biochemical testing, coaggregation, and autoaggregation.* **Findings:** *There were 7 LAB isolates obtained, namely isolates with codes GNK1, GNK3, GNR2, GNR4, GUR1, GUR3, and GUR5. Isolate GUR3 can inhibit E. coli with the highest inhibition zone of 21.31 ± 3.98 mm. In the antibacterial test against S. typhi, 4 isolates had an inhibitory activity with the highest inhibition diameter by isolate GNK3 of 28.23 ± 3.91 mm. LAB isolates from gatot have the ability to autoaggregate and coaggregate against E. coli and S. typhi. There are 5 isolates that show inhibitory activity against E. coli, namely GNK1, GNK3, GNR2, GNR4, GUR1, and GUR3. Inhibitory activity against S. typhi is shown by 4 LAB isolates, namely GNK1, GNK3, GNR2, and GNR4.* **Contribution:** *This research shows that LAB isolates from gatot have potential as probiotic agents with significant antibacterial activity against E. coli and S. typhi, so it can be developed as a natural alternative to control intestinal pathogens.*

Keywords: *Antibacterial compounds, Escherichia coli, Gatot, Lactic acid bacteria, Salmonella typhi*

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INTRODUCTION

Lampung Province is the number 1 province in Indonesia for cassava production. Based on data from the Food Security, Food Crops, and Horticulture Service, Lampung Province produced 6,719,088 tons of cassava in 2023 (Dinas Ketahanan Pangan, 2023). In addition to being processed into food raw materials, cassava can be processed using fermentation technology to increase its nutritional value. Gatot is one of the traditional foods resulting from spontaneous fermentation with cassava as the basic ingredient. During the fermentation process, there will be changes in the physical properties and nutritional content of a food ingredient as a result of the metabolic activity of microorganisms (Wardani et al., 2021). In gatot, there was a spontaneous solid fermentation process that resulted in a change in the color of cassava to blackish and a chewy texture. This texture is obtained from the process of starch hydrolysis by lactic acid bacteria (LAB) which occurs during spontaneous submerged fermentation (Astriani et al., 2018). Some types of LAB found in gatot are *Lactobacillus plantarum-pentosus*, *Lactobacillus plantarum, Lactobacillus fermentum*, and *Pediococcus* sp. (Febriana et al., 2021). In addition, homofermentative LAB, namely *Lactobacillus manihotivora* and heterofermentative BAL, namely *Lactobacillus fermentum*, *Brevibacillus brevis*, and *Bacillus liceniformis* were also found in gatot (Suciati & Safitri, 2021).

LAB can act as probiotics because it can survive in acidic pH in the stomach and live in colonies to balance the digestive microflora. In addition, LAB can produce antimicrobial compounds such as acetic acid, lactic acid, bacteriocins, CO_2, and H_2O_2 which can eliminate the presence of pathogenic microbes in the digestive tract (Suciati & Safitri, 2021). In addition, the formation of colonies between LAB isolates as probiotic candidates is very important to form a strong defense on the walls of the digestive tract so that pathogenic bacteria do not attach directly to the digestive tract (Priadi et al., 2020).

Several studies have been conducted on the potential of LAB from fermented foods as probiotic agents, but exploration of LAB from cassava tape as a probiotic agent has not been widely done. Research conducted by Rahmah et al., (2021) obtained 4 LAB isolates from cassava tape, and these isolates are acid-resistant, so they have the potential to be used as probiotics. Afifah et al., (2023) also isolated LAB from cassava tape and obtained 2 isolates that had LAB characteristics. LAB from fermented foods is also known to act as an agent for improving the profile of the digestive tract microbiota. Research by Kusuma et al., (2021) showed that LAB from fermented soybean tempeh can significantly affect blood glucose levels and increase the diversity of digestive tract microbiota such as Firmicutes, so it can be used as a treatment agent for *diabetes mellitus* patients. Febriana et al., (2021) isolated LAB from cassava gatot from Yogyakarta and obtained 6 isolates with the genus *Pediococcus* sp., *Lactobacillus fermentum, Lactobacillus plantarum, Lactobacillus plantarum-pentosus*. BAL testing was only carried out on food product contamination bacteria and has not been tested on pathogenic bacteria in the digestive tract. Based on Febriana et al., (2021), LAB isolates from cassava gatot are known to be able to inhibit the growth of *Bacillus cereus* with the largest inhibition zone of 1.87 ± 0.67 cm^2 and *Aspergillus flavus* with the largest inhibition zone of 3.83 ± 0.73 cm^2.

The ability of LAB to produce bacteriocins or antimicrobial compounds allows it to be used as a probiotic that suppresses the growth of pathogenic microbes in the digestive tract. The pathogenic bacteria that most often cause digestive disorders are *Escherichia coli* and *Salmonella typhi*. Contamination of *E. coli* to the body that has exceeded the maximum limit can cause health problems in the digestive system such as *Gastroenteritis, Diarrhea*, and *Meningitis*. When infecting the digestive tract, *Salmonella typhi* reproduces and produces enterotoxins that will affect the secretion of water and electrolytes, causing diarrhea (Kurnia et al., 2020). This study explores BAL isolates from cassava gatot and tests their potential in inhibiting *Escherichia coli* and *Salmonella typhi*.

METHOD

Isolation and Characterization of Lactic Acid Bacteria (LAB) from Gatot Cassava

This research uses home production gatot in Kalianda, South Lampung, and from Jatimulyo, South Lampung. LAB was isolated from soaked gatot and dried gatot. A total of 1 gram of smooth Gatot sample was added with 9 ml of 0.85 % NaCl. The mixture of samples and 0.85 % NaCl was then homogenized using a vortex to obtain a dilution of 10^{-1}. Dilution was carried out in stages until a dilution series of 10^{-6} was achieved. Samples from the 10^{-5} and 10^{-6} dilution series were taken as much as 100 µl and transferred using the pour plate method into a petri dish containing De Man Rogosa Sharpe Agar (MRSA) medium. Furthermore, each petri dish containing the culture was incubated at 37 ºC for 48 hours (Kurnia et al., 2020). The single lactic acid bacteria colony that grows will be purified using the streak plate method on MRSA medium and incubated again for 48 hours at 37 ºC (Febriana et al., 2021). Each pure isolate was inoculated into MRSA media enriched with 0.5 % $CaCO_3$ for selection. Incubation was carried out for 48 hours at 37 ºC. The isolate that formed the best clear zone was selected to proceed to the next research procedure. The obtained LAB isolates were characterized based on colony morphology, Gram staining, and biochemical testing. Biochemical testing included TSIA test (Ismail et al., 2017), citrate test (Manalu et al., 2020), motility test (Giyatno & Retnaningrum, 2020), and fermentation type test (Ismail et al., 2017).

Gram staining

To observe cell morphology using the gram staining technique, the slide was sterilized using 70 % alcohol. Then one loop of pure lactic acid bacteria isolate was taken and smeared on the slide that had previously been dripped with distilled water. The preparation was then fixed by passing it several times over a Bunsen flame. Next, the bacterial isolate was given two drops of crystal violet dye and left for 1 minute. After 1 minute, the bacterial isolate was rinsed with distilled water and dried, then given 1-2 drops of iodine and left for one minute. After drying, the isolate was rinsed with distilled water and dried again. The isolate was dripped with alcohol for 30 seconds, then rinsed with distilled water and dried. Furthermore, in the final stage of staining, the isolate was given 1 drop of safranin, left for 30 seconds, rinsed using distilled water and dried. The bacterial isolate was then observed for its cell shape and

color under a microscope. The purple color indicates a group of gram-positive bacteria, while the red color indicates a group of gram-negative bacteria (Ismail et al., 2017).

Coaggregation test

A coaggregation test is conducted to determine the ability to aggregate or form colonies with other bacteria such as between isolates or with pathogenic bacteria. The formation of colonies between isolates as probiotic candidates is very important to form a strong defense on the walls of the digestive tract so that pathogenic bacteria do not stick directly in the digestive tract (Priadi et al., 2020). Each LAB isolate was grown on De Man Rogosa Sharpe Broth (MRSB) medium, and the test bacteria were grown on Nutrient Broth (NB) media. Both were incubated at 37 ºC for 18 hours, then harvested by centrifugation for 20 minutes at 3500 rpm. The precipitate formed was taken and washed twice using Phosphate Buffer Saline (PBS) solution. After washing, the precipitate was suspended in a PBS solution. Each LAB isolate suspension was taken as much as 2 ml and mixed with 2 ml of test bacterial suspension in one tube. The control tube contained each single bacterial suspension that was not mixed. Then the absorbance was measured using a spectrophotometer UV-Vis at a wavelength of 600 nm (Panjaitan et al., 2020).

Autoaggregation test

The autoaggregation property shows the ability of bacteria to form aggregates or colonies with each other or similar strains (Mulianto et al., 2022). One of the requirements for probiotics is having autoaggregation ability so that they can survive and form colonies in the digestive tract. Each isolate was grown on MRSB media at 37 ºC for 18 hours. For cell harvesting, centrifugation was carried out for 20 minutes at 3500 rpm (Panjaitan et al., 2020). Furthermore, the sediment was taken and washed using PBS twice. To determine the nature of autoaggregation, initial absorbance measurements were carried out at hour 0 and final absorbance at hour 5 of incubation at room temperature. The bacterial suspension at the top was taken as much as 0.1 mL and put into PBS 3.9 mL to measure the absorbance value using a spectrophotometer with a wavelength of 600 nm (Panjaitan et al., 2020).

Antibacterial test

Lactic acid bacteria isolate cultures were subcultured on MRSA media for 48 hours at 37 ºC. Furthermore, the subculture results were transferred to MRSB media and homogenized using a vortex. Then the subculture was incubated again for 48 hours. After the bacteria grew, centrifugation was carried out to obtain ready-to-use supernatant. Pure pathogenic bacteria that had previously been subcultured on NB media were taken as much as 50 µL and inoculated on Nutrient Agar (NA) media and flattened using an L rod. Antibacterial testing was carried out using the Kirby Bauer disc method, namely by placing disc paper (diameter 6 mm) that had previously been dripped with 20 µL of supernatant from BAL. Aquades was used as a negative control, while the antibiotic Ciprofloxacin was used for the positive control. Furthermore, the test plate was incubated at 37 ºC for 24 hours. The clear zone formed as a form of BAL

inhibition of the test bacteria was measured and then compared with the positive control and negative control (Alfinda et al., 2022).

RESULT AND DISCUSSION

Isolation and Purification of Lactic Acid Bacteria

Isolation of Lactic Acid Bacteria was carried out on 2 types of gatot samples, namely industrial and home-made gatot. Based on the isolation results, 7 LAB isolates were obtained (Table 1) which produced clear zones on MRSA media added with $CaCO_3$.

Tabel 1. Lactic Acid Bacteria Isolate from Gatot Cassava

No	Isolate	Shape	Edge	Elevation	Colour
1	GNK1	*Circular*	*Entire*	*Convex*	White
2	GNK3	*Circular*	*Entire*	*Convex*	White
3	GNR2	*Circular*	*Entire*	*Convex*	White
4	GNR4	*Circular*	*Entire*	*Raised*	Yellowish white
5	GUR1	*Circular*	*Entire*	*Convex*	White
6	GUR3	*Circular*	*Undulate*	*Convex*	White
7	GUR4	*Circular*	*Entire*	*Convex*	White

Based on Aýun et al., (2023), The composition of the bacterial community in fermented foods can be influenced by the manufacturing process, raw materials or production environment. This is in line with research of Efriwati et al., (2013) who examined tempeh samples from two home industries with different production methods. The amount of LAB in tempeh produced with two boiling processes was less than tempeh produced with one boiling process.

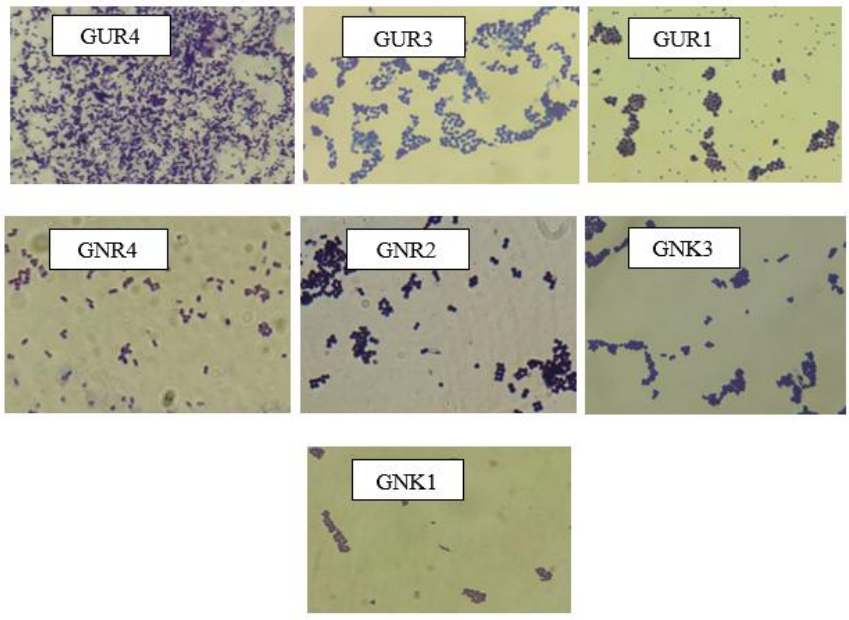

Figure 1. Gram staining results of BAL isolates from Gatot at 1000× magnification

The characterization results of the seven LAB isolates are in accordance with the general characteristics of LAB. Lactic acid bacteria are included in the group of gram-positive bacteria with round (Coccus) or rod-shaped (Cocobacili, Diplobacili, Monobacili, Palisades, and Streptobacili) (Wasis et al., 2019). The results of Gram staining on the seven selected isolates showed that the seven isolates were classified into the gram-positive bacteria group with bacillus or rod-shaped cells for isolate GUR4 and coccus or round cell shapes for isolates GUR3, GUR1, GNR4, GNR2, GNK3, and GNK1 (Figure 1).

Characterization of Lactic Acid Bacteria Isolates

In general, lactic acid bacteria (LAB) are characterized by being gram-positive, round or rod-shaped, catalase-negative, non-motile, and having two types of fermentation, namely heterofermentative and homofermentative (Hairunnisa, 2019). The catalase test results showed that there were 5 isolates that were catalase negative because they did not produce the catalase enzyme to break down hydrogen peroxide into water and oxygen. However, there were 2 isolates that showed positive catalase results, namely isolates GNR4 and GUR3. The general nature of LAB is catalase negative and non-motile, but there are several LAB that are catalase positive and motile, namely LAB from the genus *Bacillus* sp. (Alfinda et al., 2022). Motility tests conducted on the seven isolates showed that the seven BAL isolates were non-motile. Isolate growth only occurred in the puncture area and did not form a network around the puncture.

Tabel 2. Results of morphological characterization and biochemical tests of BAL

No	Isolate	Gram Staining	Biochemical Test				
			TSIA	Sitrat	SIM	Catalase	Fermentation Type
1	GNK1	Gram +	A/A	Negatif	Non motil	Negative	Heterofermentative
2	GNK3	Gram +	A/K	Negatif	Non motil	Negative	Homofermentative
3	GNR2	Gram +	A/A	Negatif	Non motil	Negative	Homofermentative
4	GNR4	Gram +	A/K	Negatif	Non motil	Positive	Heterofermentative
5	GUR1	Gram +	A/A	Negatif	Non motil	Negative	Homofermentative
6	GUR3	Gram +	A/A	Negatif	Non motil	Positive	Homofermentative
7	GUR4	Gram +	A/A	Negatif	Non motil	Negative	Homofermentative

Description: Alkaline/K (red) and Acid/A (yellow)

The fermentation type test of the seven BAL isolates showed homofermentative results in 5 isolates, namely GNK3, GNR2, GUR1, GUR3 and GUR4. While 2 of the isolates showed heterofermentative fermentation types which were marked by the formation of CO_2 gas bubbles in the Durham tube. Based on Detha et al., (2019) Lactic Acid Bacteria have 2 types of fermentation, namely homofermentative which produces most of the lactic acid as a fermentation product and heterofermentative type which produces the final fermentation products in the form of lactic acid, acetic acid, ethanol and CO_2.

The results of the autoaggregation test (Figure 4) showed that the seven isolates had autoaggregation ability with the highest autoaggregation percentage in isolate GNK1, which was 61.97 % and the lowest percentage in isolate GNK3, which was 24.95 %.

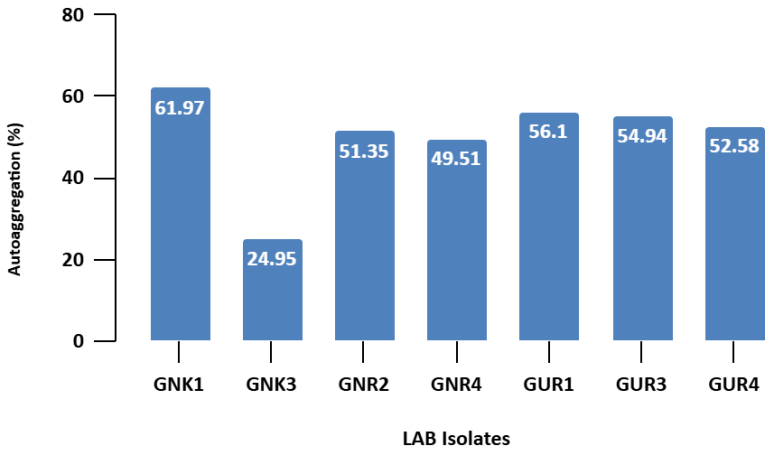

Figure 2. Percentage of autoaggregation of BAL isolates

Bacteria with an autoaggregation percentage below 10% are said to have no autoaggregation ability and are said to have good autoaggregation if they reach 40 % (Wang et al., 2010; Asnita & Meryandini, 2023). Based on the statement, the seven LAB isolates from Gatot have autoaggregation ability. As many as 6 isolates out of a total of 7 isolates have good coaggregation ability with a coaggregation percentage exceeding 40 %. Autoaggregation indicates the ability of a bacterium to attach or form a colony with its peers or similar strains. The autoaggregation properties of lactic acid bacteria (LAB) as probiotic candidates are needed to protect the intestinal mucosa from pathogenic bacteria. Probiotic colonies formed on the intestinal mucosa will act as a barrier so that pathogenic bacteria cannot attach directly to the intestinal mucosa (Darmastuti et al., 2021).

The results of the coaggregation test showed that there were 3 LAB isolates from Gatot that were able to coaggregate with *E. coli* and *S. typhi* bacteria, namely GNR2, GNR4, and GUR1, while the other 3 isolates were able to coaggregate with one of the test bacteria and there was 1 isolate, namely GNK3, which did not have the ability to coaggregate with both test bacteria (Figure 3).

The ability of coaggregation is one of the mechanisms of inhibition of pathogenic bacteria in the digestive tract. The ability of coaggregation is correlated with the ability to prevent batogenic bacteria from colonizing directly in the digestive tract. In addition, lactic acid bacteria (LAB) that are able to form aggregates or coaggregate with pathogenic bacteria can also directly expose their antimicrobial compounds (Janković et al., 2012). Based on Panjaitan et al., (2020), The coaggregation ability can be said to be strong if it reaches a coaggregation value of 30 %. Based on the results of the coaggregation test (Figure 3), it is known that there are differences in the coaggregation ability between the seven isolates. It is known that there are 3 isolates that only show coaggregation ability in *E. coli*, namely isolates GNK1 (18.8 %), GUR3 (9.85 %) and GUR4 (17,46 %). While the other 3 isolates, namely GNR2 (35.82 %), GNR4 (16.98 %), and GUR1 (52.49 %) showed better coaggregation ability in *Salmonella typhi*.

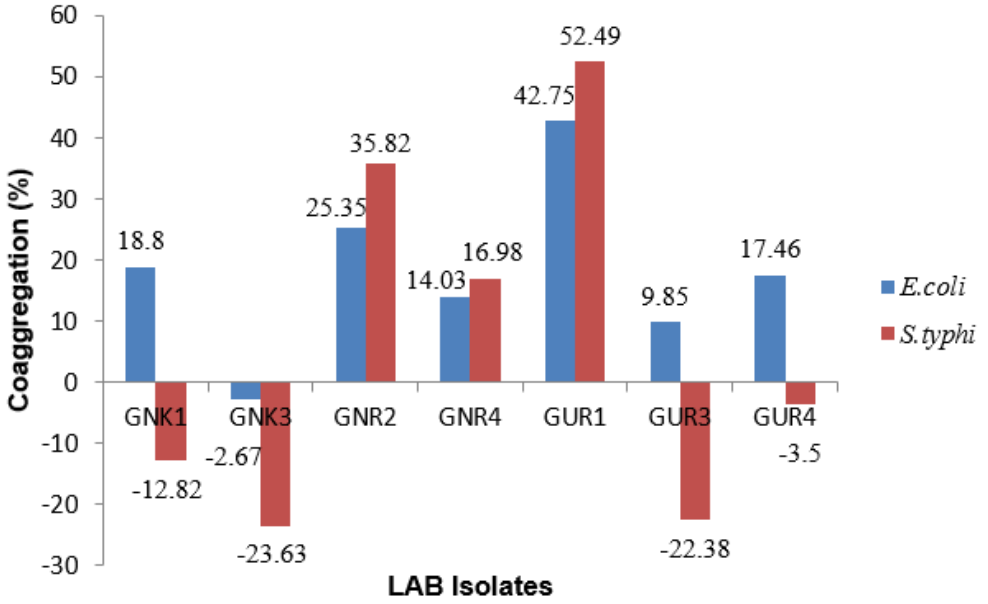

Figure 3. Percentage of coaggregation of BAL isolates against *E. coli* and *S. typhi* bacteria

There are several factors that affect the coaggregation value, namely strain specificity, inhibitors, and incubation time. This is in accordance with research conducted by Kumari et al., (2022), where there is a difference in coaggregation ability between *Lactobacillus rhamnosus* and *Lactobacillus reuteri* against *E. coli* and *S. typhi*. The *L. rhamnosus* species showed a coaggregation value against *E. coli* of 25.7 % and *S. typhi* of 32.5 %. The *L. reuteri* species showed a coaggregation ability against *E. coli* of 28.1 % and *S. typhi* of 17.8 %. The difference in coaggregation value between the two species against pathogenic bacteria shows that the coaggregation ability is strain specific (Bhushan et al., 2020; Kumari et al., 2022). Coaggregation occurs due to the interaction between sticky proteins called adhesins on the surface of a bacterial cell with receptors in the form of complex sugars on the surface of other bacterial cells (Rickard et al., 2024).

In addition, the presence of inhibitor compounds can inhibit bacterial coaggregation. In a study conducted by Stevens et al., (2015), regarding coaggregation inhibition, there were 10 out of 15 pairs of bacterial coaggregation tests that were inhibited by sugar and amino acids. The coaggregation value will increase as the incubation time increases (Priadi et al., 2020). This statement is in accordance with research conducted by Hameed & Salman (2023), where the percentage of coaggregation of *Lactobacillus garviae* (Lc1) bacteria against *Escherichia coli* was -11.90 % at 4 hours of incubation but the percentage of coaggregation increased to 70.21 % at 24 hours of incubation.

Antibacterial activity of BAL metabolites against *E. coli* and *S. typhi*

There were 6 isolates that showed inhibitory activity against *E. coli* with the highest inhibition of 21.31 ± 3.98 mm in the GUR3 treatment. While in the *S. typhi* test bacteria there were 4 isolate treatments that showed inhibitory activity with the

highest inhibition of 28.23±3.91 mm in the GNK3 treatment. Differences in inhibitory activity can occur due to differences in defense mechanisms between the two test bacteria.

The results of antibacterial tests of isolates that showed inhibitory activity with a fairly wide diameter against *E. coli* were different from isolates that showed inhibitory activity against *S. typhi*. Isolates that showed inhibitory activity with a fairly wide diameter against *E. coli* were GNK1 (19.86±2,95 mm), GUR1 (18.63 ± 1.54 mm) and GUR3 (21.31±3.98 mm). Meanwhile, isolates that showed inhibitory activity against *S. typhi* were GNK3 (28.2±3.91 mm) and GNR2 (20.01±1.84 mm). These differences can occur due to differences in defense mechanisms between *E. coli* and *S. typhi*. In a study conducted by Rezania et al., (2011), It is known that there are differences in the structure of lipopolysaccharides between *Escherichia coli* and *Salmonella typhi*. Tortora et al., (2007) Lipopolysaccharides are one of the components of the cell walls of gram-negative bacteria that function as a barrier or protector for bacteria, one of which is from antibacterial molecules.

Tabel 3. Antibacterial activity of BAL isolate metabolites against *E. coli* and *S. typhi*

Tested Bacteria	Treatment	Average Diameter of Inhibition Zone ± SD	Category
Escherichia coli	K+	48.61±6.55	Very Strong
	K-	0±0	No Inhibition
	GNK1	19.86±2.95	Strong
	GNK3	2.73±1.48	Weak
	GNR2	1.48±1.16	Weak
	GNR4	1.95±1.45	Weak
	GUR1	18.63±1.54	Strong
	GUR3	21.31±3.98	Very Strong
	GUR4	0±0	No Inhibition
Salmonella typhi	K+	50.16±2.07	Very Strong
	K-	0±0	No Inhibition
	GNK1	1.85±0.26	Weak
	GNK3	28.23±3.91	Very Strong
	GNR2	20.01±1.84	Very Strong
	GNR4	1.05±0.22	Weak
	GUR1	0±0	No Inhibition
	GUR3	0±0	No Inhibition
	GUR4	0±0	No Inhibition

Fermentation of energy substrates carried out by lactic acid bacteria (LAB) can produce various components that act as antimicrobials such as hydrogen peroxide, lactic acid, bacteriocins and several organic acids (Ismail et al., 2017). Lactic acid produced by LAB can inhibit the growth of other bacteria by a pH reduction mechanism. Organic acids such as lactic acid have bactericidal properties if their concentration exceeds 0.2 % at pH 4.5. Other organic acids that are also produced by LAB and act as antibacterial compounds with lactic acid include acetic acid, propionate and butyrate (Iacob et al., 2019).

Inhibition by bacteriocins occurs through the formation of pores in the target bacterial cell membrane which will disrupt the permeability of the cytoplasmic

membrane (Pratiwi et al., 2022). Positively charged bacteriocins will form electrostatic interactions with their receptors, namely bacterial cell membrane lipids that have a negative charge. The binding process of bacteriocins to bacterial cells will result in instability of the bacterial cell membrane so that pores or holes can form in the cell membrane. The pores that are formed can result in leakage of metabolites from within the cell, a decrease in cellular pH, the entry of substances from outside the cell so that it can inhibit bacterial growth or even cause death (Firdaus et al., 2020). In addition to bacteriocins and lactic acid, hydrogen peroxide produced by LAB also has bactericidal type antibacterial properties by damaging nucleic acids, proteins and lipids (Murphy & Friedman, 2019).

CONCLUSION

LAB isolates from Gatot have the ability to autoaggregate and coaggregate against *Escherichia coli* and *Salmonella typhi*. There are 5 isolates that show inhibitory activity against *Escherichia coli*, namely GNK1, GNK3, GNR2, GNR4, GUR1, and GUR3 with the highest average inhibition diameter of 21.31 ± 3.98 mm (GUR3). Inhibitory activity against *Salmonella typhi* is shown by 4 LAB isolates, namely GNK1, GNK3, GNR2, and GNR4 with the highest average inhibition diameter of 28.23±3.91 mm (GNK3). This study provides a scientific contribution by revealing the potential of LAB isolates from gatot as a source of probiotics that are effective in inhibiting the growth of *E. coli* and *S. typhi*, so that it can support the development of natural antimicrobial agents based on local fermented foods.

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