AL-KAUNIYAH: Jurnal Biologi, 18(1), 2025, 168-181
Website: http://journal.uinjkt.ac.id/index.php/kauniyah
P-ISSN: 1978-3736, E-ISSN: 2502-6720

BACTERIOCIN ACTIVITY OF LACTIC ACID BACTERIA FROM GIANT PRAWN (*Macrobrachium rosenbergii*)

AKTIVITAS BAKTERIOSIN DARI BAKTERI ASAM LAKTAT ASAL UDANG GALAH (Marobrachium rosenbergii)

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Submitted: 19 March 2024; Revised: 27 June 2024; Accepted: 14 August 2024

Abstract

Food is one of the necessities of life. Food is often added with preservatives such as chemicals that harm human health. One of the safe natural preservatives is bacteriocin compounds. Bacteriocins can be produced by lactic acid bacteria (LAB). These bacteriocins have known as Generally Recognized as Safe (GRAS) status. This study aimed to isolate and identify BAL from the digestive tract of giant shrimp (*Macrobrachium rosenbergii*), as well as test the ability of the bacteriocin produced to the proteolytic enzyme, temperature, pH, and salt. The research methods used were bacterial isolation, bacterial characterization, hemolysis test, bacteriocin antibacterial activity tests, proteolytic enzyme influence tests on bacteriocin activity, temperature, pH, and salt content tests on bacteriocin activity, and antibiotic tests. The research results showed that there were 37 LAB isolates and there were 7 isolates that produced bacteriocins. The LAB isolated from the digestive tract of giant prawns is Gram-positive bacteria in the form of bacilli, catalase-negative, gamma hemolytic, methyl red positive, and homofermentative. The bacteriocins can inhibit the pathogenic bacteria *Staphylococcus aureus* and *Escherichia coli* and be degraded by the Protease-K enzyme. Moreover, the bacteriocins have the characteristics of being stable at acid to neutral pH (pH 2–7), stable at low and high temperatures (4–100 °C), and stable under conditions with a salt content of 2–6.5%. The results of the identification of LAB belonged to the *Lactobacillus* genus.

Keywords: Bacteriocins; Giant prawns; Lactic acid bacteria; *Lactobacillus*; *Macrobrachium rosenbergii*

Abstrak

Makanan merupakan kebutuhan pokok dalam kehidupan sehari-hari manusia. Makanan sering kali ditambahkan bahan pengawet seperti bahan kimia yang berpengaruh buruk terhadap kesehatan manusia. Salah satu alternatif bahan pengawet alami yang aman bagi kesehatan manusia adalah senyawa bakteriosin. Bakteriosin dapat dihasilkan dari bakteri asam laktat (BAL). Bakteriosin yang diproduksi oleh BAL sudah berstatus Generally Recognized as Safe (GRAS). Penelitian ini bertujuan untuk mengisolasi dan mengidentifikasi BAL dari saluran pencernaan udang galah (Macrobrachium rosenbergii), serta menguji kemampuan bakteriosin yang dihasilkan terhadap enzim proteolitik, suhu, pH dan kadar garam. Metode penelitian yang dilakukan adalah isolasi bakteri, karakterisasi bakteri, uji hemolisis, uji aktivitas antibakteri bakteriosin, uji pengaruh enzim proteolitik, suhu, pH dan kadar garam terhadap aktivitas bakteriosin. Hasil isolasi terdapat 37 isolat BAL dan 7 isolat yang menghasilkan bakteriosin. BAL yang diisolasi dari saluran pencernaan udang galah merupakan bakteri Gram positif berbentuk basil, katalase negatif, gamma hemolisis, methyl red positif dan homofermentatif. Bakteriosin mampu menghambat bakteri patogen Staphylococcus aureus dan Escherichia coli, dapat didegradasi oleh enzim Protease-K, stabil pada pH asam hingga netral (pH 2–7), stabil pada suhu rendah maupun tinggi (4°–100 ℃) dan stabil pada kondisi dengan kadar garam 2–6,5%. Hasil identifikasi BAL dari usus udang galah yaitu bakteri termasuk dalam Genus Lactobacillus.

***Kata Kunci**: Bakteriosin; Bakteri asam laktat, Lactobacillus, Macrobrachium rosenbergii; Udang galah*

Permalink/DOI: http://dx.doi.org/10.15408/kauniyah.v18i1.38124

INTRODUCTION

Food is a basic need in human daily life (Setiawan et al., 2021). Food is often added with preservatives to extend the shelf life by inhibiting the growth of food spoilage bacteria. Commonly, chemical compounds are used as preservatives. However, the use of chemicals has dangerous health issues (Ramadhani, 2021). Misuse of preservatives may be immediate or may be harmful in the long run if humans have constant exposure to excessive doses or accumulations (Inetianbor et al., 2015). Currently, public awareness of food safety is increasing, so alternative natural preservatives are needed. Natural preservatives are safer and do not harm human health (Wahongan et al., 2021; Hernández-González et al., 2021). One of the natural preservatives is bacteriocin compounds (Kasi et al., 2017; Hernández-González et al., 2021; Yap et al., 2022).

Bacteriocins are ribosomally synthesized proteins or short polypeptides that have the potential as natural preservatives (biopreservatives) and have an antimicrobial activity that can inhibit the growth (bacteriostatic) and even kill other bacteria (bactericidal effects) (Prudêncio et al., 2015; Suwayvia, 2017; Zimina et al., 2020; Hernández-González et al., 2021; Yap et al., 2022). The use of bacteriocins as biopreservation because they are easily digested by enzymes in the digestive tract as well as they are not toxic and safe for the environment (Silva et al., 2018; Todorov et al., 2022). Moreover, they are resistant to heat or cold, easily adapt to their environment so they are stable to withstand food manufacturing processes involving low or high pH conditions and do not change the taste of food (Hernández-González et al., 2021; Todorov et al., 2022).

Bacteriocins can be produced from lactic acid bacteria (LAB) (Hernández-González et al., 2021; Yap et al., 2022). Bacteriocins produced by LAB are known as Generally Recognized As Safe (GRAS) for food additives (Plavec & Berlec, 2020). LAB can produce lactic acid from carbohydrates. LAB can be isolated in abundance from the digestive tract of shrimp (Mohamad et al., 2020). Shrimps are omnivorous scavengers or detritus and carnivores that eat small *Crustaceans, Amphipods*, and *Polychaetes*, thus allowing large amounts of LAB in their digestive tract (Romadhon et al., 2012). According to Buntin et al. (2008), fresh water and seawater are sources of LAB so the intestines of shrimp and other animals in water are a natural reservoir for LAB.

Some previous studies about the isolation of bacteriocin-producing lactic acid bacteria from giant prawns (*Macrobrachium rosenbergii)* have been conducted (Wardani et al., 2015; Feliatra et al., 2018). In those previous studies, the bacteriocin obtained is intended as a feed probiotic that can inhibit pathogenic bacteria that cause disease in aquaculture such as *Vibrio alginolyticus*, *Pseudomonas stutzeri* and *Aeromonas hydrophila*. In several studies that have been carried out, bacteriocins obtained from giant prawns have not been tested for the effect of proteolytic enzymes, temperature, pH, and salt content on bacteriocin activity. Giant prawns are estuary biota which include shrimp native to Indonesia (Adibrata et al., 2022). Giant prawns are the most popular freshwater prawns because they are large in size and have a high protein content and are a target for exports and large restaurants because they have a delicious taste and high nutritional content (Adawyah et al., 2017; Fajrilian, 2017; Manurung et al., 2018). The study about the ability of bacteriocins from giant prawns to inhibit food spoilage pathogenic bacteria has never been reported. Therefore, this study aimed to isolate and characterize bacteriocin-producing LAB from the digestive tract of giant prawns (*M. rosenbergii).* This study evoked the potential LAB as bacteriocin producers that play a role as a preservative.

MATERIALS AND METHODS

Isolation and Purification of Lactic Acid Bacteria (LAB)

A total of 5 adult giant prawns with sizes approximately 10–20 cm which were obtained by fishing from the river in Kotawaringin Village, Puding Besar District, Bangka Regency were used as samples. Samples of live giant prawns were put in an ice box and taken to the laboratory to be identified. Shrimp were identified using Short's identification key (de Bruyn, 2005). Before the isolation of LAB, some preparations were done such as sterilizing the surface of shrimp using 70% ethanol. Then the shrimp was sliced from the dorsal to the anus with a sterile knife, then the intestines are removed using a sterile knife. The shrimp intestines were crushed and then placed in a petri dish.

A total of 1 g of shrimp intestines were diluted with sterile water and serial dilution was conducted. One hundred µL of 10^{-1} and 10^{-2} dilutions spread onto MRS agar with $CaCO_3$. The clear zone around the colony showed LAB (Azahar et al., 2018). The LAB was purified using MRS agar.

Characterization of Lactic Acid Bacteria (LAB)

Several methods were used to characterize isolated LAB, namely colony characterization and cell characterization. The observed colony characters include colony shape, margins, color, and elevation. The observed cell characteristics include Gram staining, biochemical tests (catalase test, TSIA test, MR test, motility test, and fermentative type test), and physiological tests (temperature, pH, salt content). All of the characters of isolates LAB were analyzed using a Multi-Variate Statistical Package to reduce the number of isolates for further testing.

Hemolysis Test

The hemolysis test was carried out by testing the safety of LAB isolates using blood agar according to Thakkar et al. (2015) with slight modification, namely sheep blood was replaced with 5% remaining human blood from the transfusion. One loop of BAL isolates was taken and then inoculated into blood agar media. Then they were incubated at 37 °C for 2 × 24 hours. The clear zone around the colony after incubation showed a positive result for beta hemolysis.

Lactic Acid Bacteria (LAB) Resistance Test to Antibiotics

The antibiotics used were ampicillin, tetracycline, and chloramphenicol. The standard concentration of ampicillin is 10 mg, tetracycline 30 mg, and chloramphenicol 30 mg. The test was carried out by inoculating LAB isolates in MRSA media using the pour plate method. Paper discs were soaked for 15 minutes into the antibiotic. Then, the soaking paper discs were put onto MRSA media, then incubated for 24 hours at a temperature 37 °C. The activity of the clear zone produced around the paper disc was measured using a caliper (Ahsaniyah et al., 2023).

Antibacterial Activity Test of Bacteriocins

The antibacterial activity was carried out using the disc diffusion method. LAB isolates were grown in 5.0 mL liquid MRS medium and incubated at 37 °C for 24 hours. The liquid culture was centrifuged at 4,500 rpm for 10 minutes. The filtrate was neutralized to pH 6.0 by adding 1 N NaOH solution. The filtrate was sterilized using a millipore filter with a diameter of 0.22 µm into a sterile tube to obtain an antibacterial supernatant (Sari et al., 2011). The tested bacteria for antibacterial activity were *Escherichia coli* and *Staphylococcus aureus*. The number of bacterial cells was measured using the 0.5 Mc Farland standard (approximately 1.5×10^8 CFU/mL). As much as 100 µL of each bacterium was spread onto agar. A total of 20 µL of antibacterial supernatant was dropped onto a sterile paper disc with a diameter of 6 mm. The disc papers were placed on NA media containing the tested bacteria *E. coli* and *S. aureus*. The diameter of the clear zone produced around the paper disc was measured using a caliper after incubation for 24 hours at 37 °C (Sidabutar et al., 2015).

Effect of Proteolytic Enzymes on Bacteriocin Activity

A total of 250 µL of antibacterial supernatant was mixed with 750 µL of Protease-K enzyme at a concentration of 1 mg/mL dissolved in phosphate buffer pH 7.5 then incubated for 5 hours at 37 °C. The filtrate was filtered using a millipore filter with a diameter of 0.22 µm into a sterile tube (Sari et al., 2011). A total of 20 µL of antibacterial supernatant was dropped onto a sterile paper disc with a diameter of 6 mm. The disc papers were placed on NA media containing the tested bacteria *E. coli* and *S. aureus* and followed with incubated for 24 hours at 37 °C (Sidabutar et al., 2015).

Effect of Temperature on Bacteriocin Activity

The effect of temperature on bacteriocin activity was conducted according to Saad et al. (2015) with slight modification. A total of 5 mL of crude bacteriocin supernatant was stored for 30 minutes at 4 °C in the refrigerator, 27 °C in the room, and heated at 40; 60; and 80 °C, minutes using a

thermostatic water bath at 100 °C using an oven bacteriocin activity was then tested using the disk diffusion method using *E. coli* and *S. aureus* as the tested bacteria. A total of 20 µm of antibacterial supernatant was dropped onto a sterile paper disc with a diameter of 6 mm. The disc paper was placed on NA media containing the tested bacteria *E. coli* and *S. aureus*. The diameter of the clear zone produced around the paper disc was measured using a caliper after incubation for 24 hours at 37 °C (Sidabutar et al., 2015).

Effect of pH on Bacteriocin Activity

A total of 5 mL of crude bacteriocin solution was put in different tubes. pH of each tube was adjusted to pH 3, 6, 8, and 10 using NaOH or HCl solution. After incubation for 4 hours at 37 °C, bacteriocin activity was then tested using the disk diffusion method (Kusmarwati et al., 2014). A total of 20 µm of antibacterial supernatant was dropped onto a sterile paper disc with a diameter of 6 mm. The disc paper was placed on NA media containing the tested bacteria *E. coli* and *S. aureus*. The diameter of the clear zone produced around the paper disc was measured using a caliper after incubation for 24 hours at 37 °C (Sidabutar et al., 2015).

Effect of Salt Concentration on Bacteriocin Activity

A total of 5 mL of crude bacteriocin solution in different tubes was added with NaCl with concentrations of 2; 4; and 6%, respectively. Then all tubes were incubated at 37 °C for 12 hours (Verluyten et al., 2004). Bacteriocin activity was then tested using the disk diffusion method using the indicator bacteria *E. coli* and *S. aureus*. A total of 20 µm of antibacterial supernatant was dropped onto a sterile paper disc with a diameter of 6 mm. The disc paper was placed on NA media containing the tested bacteria *E. coli* and *S. aureus*. The diameter of the clear zone produced around the paper disc was measured using a caliper after incubation for 24 hours at 37 °C (Sidabutar et al., 2015).

RESULTS

Characterization of Lactic Acid Bacteria (LAB)

A total of 37 isolates producing clear zones (data were not shown) could be isolated from 5 samples of the digestive tract of giant prawns. A clear zone forming indicated that the isolates could produce lactic acid and were suspected to be LAB. One of the isolates LAB colonies on MRS agar can be seen in Figure 1. A total of 3 isolates from 37 isolates were gram-negative bacteria and a total of 8 isolates were methyl red negative. Only 26 isolates were subjected to the MSVP test to reduce samples for further testing so that 10 taxa were obtained (data were not shown).

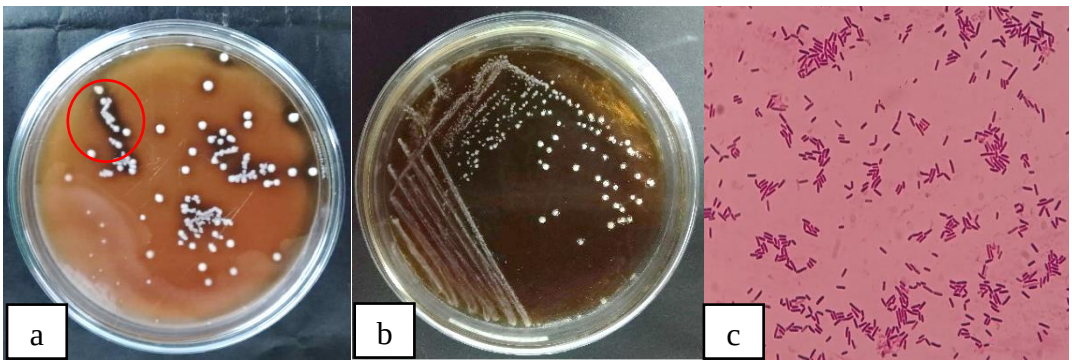

Figure 1. Bacterial colony's appearance, a colony of lactic acid bacteria LAB isolates on MRSA+ $CaCO_3$ (a), purification of one of LAB isolates (b), and Gram staining of LAB (c)

The bacteriocin test showed that 3 isolates did not produce a clear zone, so only 7 isolates were tested for the influence of proteinase, pH, temperature, and salt content. Only data for 7 isolates were shown in this article. The selected isolates were further observed for their colony and cell morphological characteristics, biochemical properties, and physiological properties. The characterization results showed that the LAB isolates were Gram-positive *Bacillus*, catalase-negative, methyl red positive, negative motility, and homofermentative. LAB isolates could grow at

temperatures of 4; 25; and 37 °C, while at 45 °C 2 isolates did not grow, namely UG5 and UG21. All isolates were able to grow at pH 2.5, 3, and 5 and were able to grow at salt content of 4 and 6.5%. The results of the characterization of LAB isolates are shown in Table 1.

Table 1. Characterization and identification of (lab) isolates

Characteristics	Isolates code						
	UG4	UG5	UG15	UG21	UG23	UG30	UG35
Macroscopic							
Shape	Circular	Circular	Circular	Circular	Circular	Circular	Circular
Margin	Entire	Entire	Entire	Entire	Entire	Entire	Entire
Elevation	Convex	Convex	Convex	Convex	Convex	Convex	Convex
Color	White	Milky White	Broken White	Broken White	Milky White	Cream	Broken White
Microscopic							
Gram staining	+	+	+	+	+	+	+
Cell shape	Bacil	Bacil	Bacil	Bacil	Bacil	Bacil	Bacil
Biochemical test							
Catalase	-	-	-	-	-	-	-
Methyl red	+	+	+	+	+	+	+
Motility	-	-	-	-	-	-	-
TSIA	++	++	++	++	++	++	++
Physiology							
Temperature 4 °	++	+++	+++	++	++	++	++
25 °	+++	+++	+++	+++	++	+++	+++
37 °	+++	+++	+++	+++	+++	+++	+++
45 °	++	-	++	-	++	+	++
pH 2.5	+	+	+	+	+	+	++
3	++	+	++	+	+	+	+
5	+++	+++	+++	+	+++	+	+
NaCl 4%	++	+	++	+	+	+	+++
6.5%	+	+	+	+	+	+	+++
Genus	*Lactobacillus*						

Note: +++= very cloudy and lots of sediment; ++= cloudy and quite a lot of sedimen; += cloudy and no sediment; -= no growth

The Hemolysis Test

The results of the hemolysis test on blood agar media did not show any clear zones around the colonies (Figure 2). These results showed no hemolytic activity for α and β-hemolysis and all isolates were negative for hemolysis or gamma hemolysis.

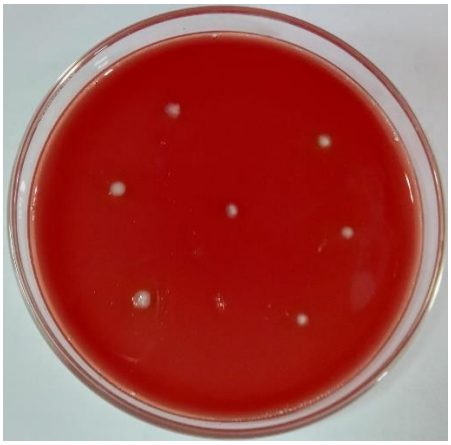

Figure 2. Hemolytic activity of lactic acid bacteria (lab) isolates on blood agar

Lactic Acid Bacteria (LAB) Resistance Test to Antibiotics

The test results showed that all isolates were resistant to the antibiotics ampicillin, tetracycline, and chloramphenicol with an inhibition zone of <15 mm (Table 2).

Table 2. Clear zone diameter of isolates of lactic acid bacteria (lab) for antibiotics

Code isolates	Clear zone diameter (mm) of lab isolates against antibiotics			
	Ampicillin	Tetracycline	Chloramphenicol	Negative control
UG4	9.63	6.11	5.80	-
UG5	9.64	5.9	3.97	-
UG15	8.29	8.14	6.39	-
UG21	9.29	6.66	4.58	-
UG23	11.48	6.02	5.78	-
UG30	8.33	6.68	4.80	-
UG35	6.94	5.33	3.60	-

Antibacterial Activity and Effect of Protease on Bacteriocins

The test was carried out using the disc diffusion method as well as *E. coli* and *S. aureus* as tested bacteria. The results showed antibacterial bacteriocin supernatants from LAB isolates were able to inhibit pathogenic bacteria as indicated by the presence of a clear zone (Figure 3, 4, & Table 3). Antibacterial supernatants were treated with proteolytic enzymes to confirm that the antibacterial supernatants produced by LAB isolates were bacteriocins. The proteolytic enzyme used is the Proteinase-K enzyme. Table 3 showed that seven isolates lost their activity in inhibiting *E. coli* as indicated by the loss of the clear zone as a result of the addition of the Proteinase-K enzyme, however, there were 2 bacteriocin antibacterial supernatants that did not lose their activity in inhibiting *S. aureus*, namely UG30 and UG35.

Table 3. Antibacterial activity of bacteriocins and effect of protease on bacteriocins

Isolates code	Antibacterial activity of lab isolates		Effect of protease to bacteriocins	
	E. coli (mm) X ± SD	*S. aureus* (mm) X ± SD	*E. coli* (mm) X ± SD	*S. aureus* (mm) X ± SD
Negative control	0 ± 00	0 ± 00	0 ± 00	0 ± 00
Chloramphenicol	7.30 ± 0.238	9.24 ± 0.421	0 ± 00	0 ± 00
UG4	9.78 ± 0.103	12.72 ± 0.033	0 ± 00	0 ± 00
UG5	9.26 ± 0.021	10.37 ± 0.078	0 ± 00	0 ± 00
UG15	9.07 ± 0.075	12.25 ± 0.163	0 ± 00	0 ± 00
UG21	9.31 ± 0.007	11.79 ± 1.032	0 ± 00	0 ± 00
UG23	9.26 ± 0.007	11.34 ± 0.229	0 ± 00	0 ± 00
UG30	8.90 ± 0.245	9.21 ± 0.332	0 ± 00	5.03 ± 0.212
UG35	9.10 ± 0.177	9.60 ± 0.069	0 ± 00	5.01 ± 0.690

Note: X= means; SD= standard deviation

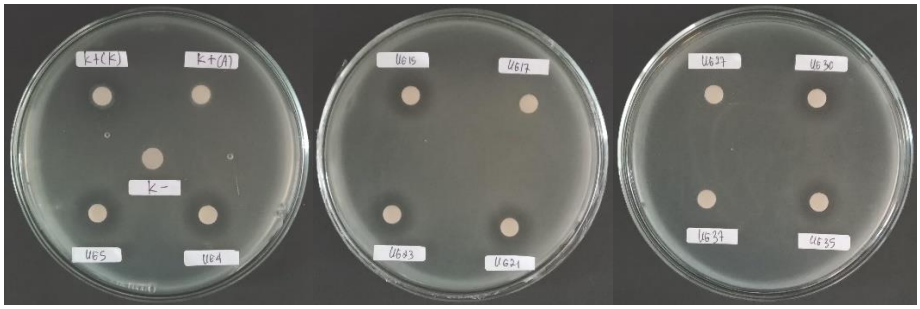

Figure 3. Antibacterial activity of bacteriocins against *E. coli* bacteria

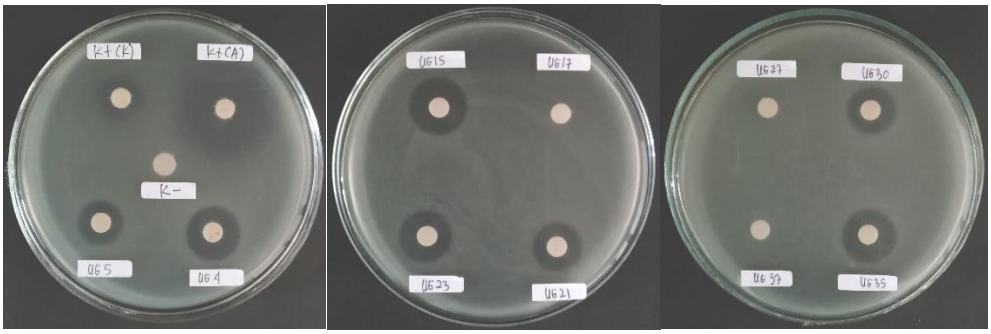

Figure 4. Antibacterial activity of bacteriocins against *S. aureus* bacteria

Effect of Temperature on Bacteriocin Activity

The temperature test results showed that the bacteriocins supernatant were able to inhibit pathogenic bacteria at temperatures of 4; 40; 60; 80; and 100 °C, as indicated by the presence of a clear zone around the disc (Table 4 & 5).

Table 4. The effect of temperature on bacteriocin activity in inhibiting *E. coli*

Isolates code	Clear zone (mm) X ± SD *E. coli*				
	4 °C	40 °C	60 °C	80 °C	100 °C
UG4	4.24 ± 0.127	4.54 ± 0.141	5.36 ± 1.193	4.89 ± 1.301	4.49 ± 0.184
UG5	4.25 ± 0.049	4.36 ± 0.014	5.38 ± 0.255	4.87 ± 0.778	3.90 ± 0.453
UG15	3.82 ± 0.212	4.28 ± 0.033	3.53 ± 0.382	3.85 ± 1.167	3.77 ± 0.559
UG21	3.64 ± 0.191	4.41 ± 0.000	4.31 ± 0.127	4.19 ± 0.177	3.56 ± 0.283
UG23	3.99 ± 0.042	4.14 ± 0.057	3.64 ± 0.198	4.21 ± 0.081	3.14 ± 0.120
UG30	4.60 ± 0.226	3.95 ± 0.071	2.89 ± 0.035	4.42 ± 0.198	2.81 ± 0.243
UG35	3.39 ± 0.177	3.93 ± 0.028	3.76 ± 0.707	4.40 ± 0.212	2.93 ± 0.205

Table 5. The effect of temperature on bacteriocin activity in inhibiting *S. aureus*

Isolates code	Clear zone (mm) X ± SD *S. aureus*				
	4 °C	40 °C	60 °C	80 °C	100 °C
UG4	9.72 ± 0.015	8.58 ± 0.184	11.36 ± 0.007	10.34 ± 0.580	9.45 ± 0.198
UG5	8.90 ± 0.495	8.34 ± 0.099	10.12 ± 0.332	10.08 ± 0.212	8.93 ± 0.156
UG15	9.64 ± 0.065	10.85 ± 1.103	10.37 ± 0.573	10.15 ± 0.509	8.48 ± 0.820
UG21	8.45 ± 0.450	9.75 ± 0.382	6.75 ± 0.173	10.40 ± 0.931	8.79 ± 0.573
UG23	8.75 ± 0.050	9.31 ± 0.014	8.85 ± 1.078	10.65 ± 0.948	8.26 ± 1.174
UG30	8.89 ± 0.310	9.20 ± 0.113	8.08 ± 0.099	9.36 ± 0.427	8.41 ± 0.262
UG35	7.44±0.190	8.84±0.035	8.53±0.781	10.90±0.877	8.10±0.410

Effect of pH on Bacteriocin Activity

The bacteriocins activity of isolates LAB was resistant category to inhibit *E. coli.* At pH 3, 6, 7, and 8, bacteriocins from all isolates showed their activities which was indicated by the presence of a clear zone around the paper disc (Table 6). Bacteriocin activity disappeared at pH 10. In terms of bacteriocin activity at pH in inhibiting *S. aureus* bacteria, all extracts were resistant category (Table 7). At pH 8 three supernatants did not produce a clear zone, namely UG4, UG5, and UG15. At pH 10 all bacteriocin supernatants lost their activity.

Table 6. The effect of pH on bacteriocin activity in inhibiting *E. coli*

Isolates code	Clear zone (mm) X ± SD *Escherichia coli*				
	pH 3	pH 6	pH 7	pH 8	pH 10
UG4	11.34 ± 0.707	9.78 ± 0.103	8.45 ± 0.049	1.90 ± 0.438	0.00 ± 0.00
UG5	10.08 ± 0.891	9.26 ± 0.021	8.44 ± 0.290	1.91 ± 0.339	0.00 ± 0.00
UG15	9.46 ± 0.891	9.07 ± 0.075	7.19 ± 0.255	1.76 ± 0.417	0.00 ± 0.00
UG21	11.30 ± 0.120	9.31 ± 0.007	7.63 ± 0.806	1.79 ± 0.247	0.00 ± 0.00
UG23	11.31 ± 0.106	9.26 ± 0.007	8.12 ± 0.276	2.68 ± 0.594	0.00 ± 0.00
UG30	11.01 ± 0.262	8.90 ± 0.245	7.62 ± 0.141	2.16 ± 0.537	0.00 ± 0.00
UG35	10.78 ± 0.417	9.10 ± 0.177	8.11 ± 0.038	0.66 ± 0.933	0.00 ± 0.00

Table 7. The effect of pH on bacteriocin activity in inhibiting *S. aureus*

Isolates code	Clear zone (mm) X ± SD *Staphylococcus aureus*				
	pH 3	pH 6	pH 7	pH 8	pH 10
UG4	13.61 ± 0.255	12.72 ± 0.033	8.78 ± 0.488	0.00 ± 0.00	0.00 ± 0.00
UG5	12.41 ± 1.541	10.37 ± 0.078	8.59 ± 0.559	0.00 ± 0.00	0.00 ± 0.00
UG15	11.47 ± 0.639	12.25 ± 0.163	7.15 ± 1.230	0.00 ± 0.00	0.00 ± 0.00
UG21	13.12 ± 0.368	11.79 ± 1.032	8.43 ± 0.240	3.89 ± 0.431	0.00 ± 0.00
UG23	11.75 ± 0.622	11.34 ± 0.229	8.97 ± 0.219	3.24 ± 0.552	0.00 ± 0.00
UG30	11.86 ± 0.064	9.21 ± 0.332	6.82 ± 0.431	1.71 ± 0.113	0.00 ± 0.00
UG35	13.08 ± 0.233	9.60 ± 0.069	7.87 ± 0.247	2.33 ± 0.141	0.00 ± 0.00

Effect of NaCl on Bacteriocin Activity

Table 8 showed that the antibacterial bacteriocins supernatant was able to inhibit the pathogenic bacteria *E. coli* and *S. aureus* at salt levels of 2; 4; and 6.5% as indicated by the presence of a clear zone around the disc.

Table 8. The effect of NaCl on bacteriocin activity in inhibiting *E. coli* and *S. aureus*

Isolates code	Clear zone (mm) X±SD					
	E. coli			*S. aureus*		
	NaCl 2%	NaCl 4%	NaCl 6.5%	NaCl 2%	NaCl 4%	NaCl 6.5%
UG4	8.20 ± 0.049	7.43 ± 0.511	5.98 ± 0.294	10.95 ± 0.113	8.34 ± 0.332	8.13 ± 0.750
UG5	7.26 ± 0.509	4.28 ± 0.905	6.29 ± 0.320	9.19 ± 0.057	4.98 ± 0.156	4.01 ± 0.287
UG15	8.92 ± 0.283	5.43 ± 0.229	6.29 ± 0.045	10.41 ± 0.905	7.03 ± 0.245	7.19 ± 1.177
UG21	7.52 ± 0.233	5.21 ± 0.141	6.63 ± 0.110	8.54 ± 0.375	7.86 ± 0.236	8.89 ± 1.520
UG23	8.34 ± 0.212	5.53 ± 0.495	8.11 ± 0.026	10.88 ± 0.134	7.41 ± 1.270	9.92 ± 1.252
UG30	8.42 ± 0.290	6.98 ± 0.201	6.84 ± 0.398	10.10 ± 0.028	8.11 ± 0.300	9.71 ± 2.253
UG35	6.95 ± 0.028	5.46 ± 0.184	5.86 ± 0.160	10.22 ± 0.481	8.45 ± 0.778	8.88 ± 1.962

DISCUSSION

Isolation and Characterization of Lactic Acid Bacteria (LAB)

Lactic Acid Bacteria (LAB) isolation was carried out by growing the bacteria on MRSA media plus 1% $CaCO_3$. Based on the isolation results, the selected bacteria can produce a clear zone. The lactic acid produced by LAB will react with $CaCO_3$ resulting in the formation of soluble calcium lactate in the MRSA medium which is characterized by the presence of a clear zone around the LAB colonies (Putri et al., 2020). Based on the characterization results of the seven lactic acid bacteria isolates, they may include the genus *Lactobacillus* which has characteristics, namely Gram-positive rod-shaped, non-motile, catalase-negative, MR positive, and homofermentative type fermentation. These characteristics are by Bergey's manual book (Holt et al., 1994) which states that the genus *Lactobacillus* has rod-shaped or cocci cells, usually short chains, Gram-positive bacteria, non-spores, immobile and facultative anaerobes. Colonies on the media are about 2–5 mm in diameter, convex in elevation, with intact edges and not pigmented. Mohamad et al. (2020) reported that LAB found in the digestive tract of giant prawns (*M. rosenbergii*) were from the genus *Lactobacillus*, *Enterococcus*, and *Lactococcus*. The genus *Lactobacillus* was found to be Gram-positive, rod-shaped, catalase-negative, does not form spores, homofermentative, and non-motile.

Characterization of Hemolysis

The hemolysis test was carried out to determine the safety of LAB through their hemolytic activity. Hemolytic activity is considered a virulence factor for pathogenic microorganisms (Aktas & Yigit, 2015). Based on the hemolysis test results, there was no hemolytic activity for α and β-hemolysis and all isolates included negative hemolysis or gamma hemolysis. Therefore, LAB isolates do not destroy red blood cells. This is by research conducted by Golshahi et al. (2021) which stated that the hemolytic activity of the genus *Lactobacillus* showed negative hemolysis results. Research by Mohamad et al. (2020) also reported that lactic acid bacteria isolated from freshwater shrimp *M. rosenbergii* had gamma hemolytic activity, indicating that BAL from these shrimps did not induce blood hemolysis.

Lactic Acid Bacteria (LAB) Resistance to Antibiotics

The results of the LAB resistance test to antibiotics showed that all LAB isolates were resistant to ampicillin, chloramphenicol, and tetracycline with an inhibitory diameter of ≤15mm. According to Sukarya et al. (2021), several types of bacteria are resistant to certain antibiotics due to the intrinsic nature of bacteria, namely that they can produce enzymes that inactivate antibiotic compounds. Ampicillin is a β-lactam antibiotic whose activity can be inhibited by the β-lactamase enzyme by degrading the compound so that bactericidal activity can be inhibited and supports bacteria to remain resistant to the antibiotic. Stefańska et al. (2021) reported that *Lactobacillus plantarum* resistance to ß-lactam antibiotics was associated with the presence of the bla gene. Dec et al. (2017) reported that *Lactobacillus* sp. is resistant to the antibiotic chloramphenicol due to the Cat gene. Cat gene codes

the gene for the production of the enzyme chloramphenicol acetyltransferase which converts chloramphenicol into inactive diacetyl chloramphenicol. Campedelli et al. (2019) also reported that in the genus *Lactobacillus* the Cat and CmIA genes were found to be located on plasmids and transportons. The Cat gene encodes chloramphenicol acetyltransferase and the CmIA gene for a specific membrane-associated transporter. These two genes will convert chloramphenicol into the inactive form of diacetyl chloramphenicol. Campedelli et al. (2019) also reported that *Lactobacillus* sp. was resistant to tetracycline due to the resistance genes tet (M), tet (S), tet (Q), and tet (W) for ribosome protection proteins. Moreover, the tet (L) and tet (P) genes were also found which play a role in the efflux pump. Resistance genes in LAB are obtained from mobile genetic elements that carry these resistance genes. The existence of a genetic code that can increase the chances of BAL cells surviving could be the reason why lactic acid bacteria are resistant to antibiotics such as ampicillin, chloramphenicol, and tetracycline.

Bacteriocins Activity

Lactic Acid Bacteria (LAB) isolates were able to inhibit the pathogenic bacteria *E. coli* and *S. aureus* as indicated by the presence of a clear zone around the paper disc (Figures 3 & 4). The diameter of the inhibition zone for *S. aureus* bacteria is on average larger than for *E. coli*. The difference in sensitivity can occur because Gram-negative bacteria have an outer membrane that acts as a barrier (Prudêncio et al., 2015), According to Hamidah et al. (2019), the cell wall of Gram-negative bacteria consists of lipoproteins, lipopolysaccharides, and peptidoglycan. The cell wall structure of Gram-negative bacteria caused difficulty in penetration by antibacterial compounds than Gram-positive bacteria. In line with Sari et al. (2016) who stated that *Lactobacillus plantarum* has bacteriocin activity which can inhibit *E. coli* by 8.07 mm and *S. aureus* by 11.43 mm. According to Ibrahim (2019), bacteriocins are ribosomally synthesized antimicrobial peptides (AMPs) or proteins from bacteria that can inhibit or kill closely related bacterial strains. LAB is a Gram-positive bacteria so the bacteriocin from LAB is more effective in inhibiting Gram-positive bacteria such as *S. aureus*.

The inhibition of the growth of pathogenic bacteria *S. aureus* and *E. coli* by bacteriocins is caused by the presence of peptides that bind to the plasma membrane through electrostatic interactions with negatively charged cytoplasmic membrane lipids or phospholipids. The hydrophobic part of the bacteriocin will enter the cytoplasmic membrane by forming a pore which results in the release of ions (especially potassium and magnesium), the loss of proton motive force (PMF), and the release of ATP and amino acids. PMF has a fundamental role in ATP synthesis, active transport, bacterial movement, and bacterial cell metabolism. Therefore, macromolecule synthesis and energy production are hampered, resulting in cell death (Mokoena, 2017).

The bacteriocin antibacterial supernatant which produced a clear zone was treated with proteolytic enzymes to confirm that the antibacterial supernatant produced by the LAB isolate was a bacteriocin. The test results showed that seven isolates lost their activity in inhibiting *E. coli*, which was indicated by the absence of clear zones as a result of the addition of the Proteinase-K enzyme (Table 3). Two bacteriocin supernatants still produced clear zones against *S. aureus*, namely from isolates UG30 and UG35, but the zones that appeared were reduced from the previous antibacterial activity. The inhibition zone that does not appear after bacteriocins are treated with proteolytic enzymes proves that the characteristics of bacteriocins are natural proteins that can be degraded by enzymes in the digestive tract and make them safe for consumption. According to Pratiwi et al. (2022), bacteriocins contain disulfide bonds. Proteolytic enzymes destroy the disulfide bonds of bacteriocins, resulting in loss of bacteriocin activity

Effect of Temperature on Bacteriocin Activity

Testing at a temperature of 4 °C was carried out to determine the activity of bacteriocins at storing temperature for bacteriocins, while testing at temperatures of 40; 60; 80; and 100 °C was carried out to see the ability of antibacterial activity of bacteriocins at high temperatures. The results of the bacteriocin antibacterial activity test at 4 °C showed that all bacteriocin antibacterial supernatants were able to inhibit pathogenic bacteria as indicated by the presence of an inhibitory

zone around the disc. It showed that the bacteriocin supernatant could be stored at cold temperatures. According to Dündar (2006), bacteriocins can maintain their antibacterial activity at a storage temperature of 4 °C because at this temperature the protease enzyme contained in bacteriocins becomes inactive.

The stability of bacteriocins to temperature is important if bacteriocins are to be used as food preservatives. According to Gavahian et al. (2019), temperature stability is an important characteristic if bacteriocins are used as natural food preservatives because several food preparation methods involve heating. If the bacteriocin is not resistant to high temperatures, it cannot be used as a preservative in products that require heating in the manufacturing process because it is feared that the bacteriocin will be damaged so that its antibacterial activity will no longer be effective in preserving food. The test results showed that the bacteriocin supernatant had antibacterial activity at temperatures of 40; 60; and 80 °C, while at 100 °C there was still a clear zone but the activity was reduced. Cheriet et al. (2023) reported that bacteriocins from LAB isolated from the intestines of whiting (*Merlangius merlangus*) and *Sparus aurata* were stable at storage temperatures of -20 °C and 4 °C, and stable during heat treatment at 60; 80; and 100 °C, but bacteriocin activity was lost after autoclaving (121 °C for 15 minutes). Heat resistance is a general characteristic of various types of bacteriocins produced by LAB (Sujana et al., 2020).

The antibacterial activity of bacteriocins is still present during heat treatment because bacteriocins are short peptides that are heat stable and the presence of certain amino acids in bacteriocins can maintain the bacteriocin structure from the effects of heat (Kusmarwati et al., 2014). According to Carlier et al. (2015), the mechanism of bacteriocin resistance to heat is related to the structure of the bacteriocin molecule in the form of peptides as well as the presence of hydrophobic areas, stable cross-links, and high glycine content. The bacteriocins that are heat stable in this study are thought to be class I and II bacteriocins. Most of the bacteriocins produced by LAB are class I and II bacteriocins which are proteins that have hydrophobic bonds with a tertiary structure that causes heat stability (Darbandi et al., 2022).

Effect of pH on Bacteriocin Activity

The pH factor is a consideration for preservatives that will be applied to food ingredients, especially ingredients with low pH conditions. The results of testing bacteriocins against pH in inhibiting *E. coli* (Table 7) showed that all bacteriocins from LAB isolates were resistant to pH 3, 6, 7, and 8. Bacteriocin activity disappeared at pH 10. The activity of bacteriocins against pH in inhibiting *S. aureus* (Table 8) showed that bacteriocins are resistant to pH 3, 6, and 7. At pH 8 three supernatants do not produce a clear zone, namely UG4, UG5, and UG15. At pH 10 all bacteriocin supernatants lost their activity. The antibacterial activity of bacteriocins is optimal at pH 6 and pH 7 but decreases at pH 8 and bacteriocin activity disappears at pH 10. Andarilla et al. (2018) stated that the effect of pH on the activity of bacteriocins produced by *Lactobacillus casei* from dried cuttlefish was stable at pH 2–6, there was a decrease in bacteriocin activity at pH 8 and bacteriocin activity disappeared at pH 10. Rasheed et al. (2020) reported that the antibacterial activity of bacteriocin from *Lactobacillus fermentum* was stable in the pH range of 2–8. Jiang et al. (2022) also reported that the antibacterial activity of bacteriocin from *Lactiplantibacillus plantarum* was stable between pH 2 to pH 6, and decreased significantly to 74% at pH 8. According to (Kusmarwati et al. (2014) the loss of bacteriocin activity in pH alkaline is caused by protein aggregation.

Effect of Salt Concentration on Bacteriocin Activity

Testing the effect of adding salt on bacteriocin activity is important because food processing often involves NaCl or table salt, so it is necessary to know first the characteristics of the bacteriocin that will be used and whether it has activity in inhibiting pathogenic bacteria when added with NaCl. Table 8 showed that bacteriocin activity was still stable after adding salt. It meant that the salt level did not affect bacteriocin activity, but bacteriocin activity was reduced if high salt levels were added. Sukmawati et al. (2022) reported that the addition of 2–10% NaCl to bacteriocins from rebon shrimp (*Acetes* sp.) samples had no effect because bacteriocins were still able to inhibit pathogenic bacteria.

Obi et al. (2018) also reported that bacteriocin with the addition of 1–4% NaCl was able to inhibit *S. aureus* and *E. coli* and there was no inhibition of pathogenic bacteria when NaCl was added 5–10%. Differences in bacteriocin stability indicate that the bacteriocins produced by each LAB have different characteristics.

CONCLUSION

Lactic acid bacteria (LAB) isolated from the digestive tract of giant prawns were identified as belonging to the genus *Lactobacillus* sp. Bacteriocins produced by LAB can be used as biopreservatives because of their ability to inhibit *Staphylococcus aureus* and *Escherichia coli* and can be degraded by the Protease-K enzyme. Moreover, these bacteriocins have the characteristics of being stable at acid to neutral pH (pH 2–7), stable at low and high temperatures (4–100 °C), and stable in conditions with a salt content of 2–6.5%.

Suggestions for this study include molecular identification of lactic acid bacteria to determine the bacteria at the species level, as well as further testing regarding the characteristics of bacteriocins and testing the application of isolated bacteriocin compounds.

ACKNOWLEDGMENTS

We express our gratitude to Bangka Belitung University and all individuals who provided support during this research.

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