Antibacterial efficacy of lactic acid bacteria and bacteriocin isolated from Dadih’s against *Staphylococcus aureus*

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**Abstract**

Bacteriocin, a peptide produced by lactic acid bacteria (LAB) widely used as a natural and safe preservative in food products. This work aimed to evaluate the characterization of bacteriocin from dadih’s LAB and to study its antimicrobial activity against *Staphylococcus aureus*. Supernatant from 12 LBA strains could inhibit *S. aureus* growth with different inhibition zones. Only nine strains showed inhibition zones of > 3.5 mm and were further evaluated for their antibacterial compounds. Three strains had antimicrobial activity derived from organic acids, especially lactic acid, and six strains had antimicrobial compounds in the form of bacteriocin. Bacteriocin strain R-55 showed the highest activity against *S. aureus*, with an average inhibition zone of 8.43 mm. The molecular weight of the purified isolated bacteriocin from the R-55 strain was 14.4 kDa. Bacteriocin obtained from *St. faecalis* subsp. liquefaciens R-55 is a promising natural preservative to prevent the growth of *S. aureus* as foodborne pathogens.

**Keywords:** bacteriocin; dadih; lactic acid bacteria; *Staphylococcus aureus*; antibacterial; natural preservative.

**Practical Application:** Bacteriocin from lactic acid bacteria for food bio preservatives.

1 Introduction

*Staphylococcus aureus* is the major pathogenic bacteria that infect humans and animals, resulting in several clinical manifestations of the pathogen, including the infection of superficial skin and soft tissue, sepsis, pneumonia, and endocarditis (Tong et al., 2015). These pathogenic bacteria are prone to be resistant to different antibiotics. Infections are prevalent in the hospital-acquired and community-acquired environment, and the treatment is still challenging to achieve because of the development of multi-drug resistant bacterial strains such as Methicillin-Resistant *Staphylococcus aureus* (MRSA) (Boucher & Corey 2008). MRSA is resistant to most β-lactam antibiotics due to the penicillin-binding protein encoded by the mecA gene (Chambers, 1997). Moreover, MRSA can epidemically spread in livestock and hospitals, societies (Ho et al., 2012; Vandendriessche et al., 2014). The risk of transmission of MRAS from animals to human attracted public health concern since the observation of the livestock-associated MRSA from pig belonging to LA-MRSA CC398 (Clonal Complex 398) was reported in Netherland early in the twentieth century (van Loo et al., 2007; Garcia-Graells et al., 2012).

This organism is robust so that it can grow on many types of food that are processed or stored incorrectly, and the infection causes diseases via the production of different enzymes and enterotoxins (SEs) or by the direct invasion and destruction of the tissues of the human body (Taddesse et al., 2014). This SEs is one of those compounds causing staphylococcal food poisoning (SFP) by *S. aureus* (Rasooly & Friedm, 2012) and consists of nine groups of heat-stable SEs, namely SEH, SEB, SEA, SEC, SEE, SED, SEG, SEJ, and SEI (Argudín et al., 2010). It was estimated that about 20-30% of the human population are carriers for *S. aureus* (Tong et al., 2015), which are coexist with human microbiota and cause abscesses and the infections of the skin (Carroll et al., 2017), respiratory system (Payne & Benninger, 2007; Vickery et al., 2019) and *Staphylococcal* foodborne disease (Kadariya et al., 2014). Heat treatment on food can kill *S. aureus*, but heating cannot deactivate SEs so that these toxins remain in the food, which in turn causes SFP (Hu & Nakane, 2014). Therefore, it is vital to stop the growth of *S. aureus* in food, which can subsequently produce toxins.

Several synthetic chemical preservatives are used commercially, including salts, sugar, butylated hydroxy toluene (BHT), butylated hydroxy-anisole (BHA), tert-butyl hydroquinone (TBHQ), nitrites, sodium nitrate, calcium propionate, and sulfite compounds such as sulfur dioxide, sodium bisulfite, disodium and potassium hydrogen (Walsh, 2007). The massive use of these synthetic preservatives in the food sector is mainly because these preservatives are commercially distributed in chemical stores. However, these chemicals are not always safe for human consumption because their overuse can cause health problems ranging from allergies and asthma to cancer (Dicks et al., 2017). Therefore, the development of safe, natural preservatives is critically needed. LABs produce various inhibitor compounds as primary and secondary metabolic end products like bacteriocins, hydrogen peroxide, and organic acids, especially lactic acid.
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(Sankar et al., 2012; Zhou et al., 2014). Bio-preservation is a concept of using LAB and its antimicrobial products, including bacteriocin, to inhibit the growth of pathogenic and spoilage microbes in food (Stiles, 1996). Bacteriocin is peptide molecules consisting of 12-100 amino acids with amphphilic characteristics, cationic net charge, synthesized, and released to act extracellularly and have activity against other bacteria (Rios et al., 2016). These peptides are considered GRAS (generally recognized as safe) and continue to interest many researchers because they have potential use in the pharmaceutical and food industries. In the food industry, bacteriocin has long been used to prevent food spoilage and foodborne diseases (Perez et al., 2014). These bacteriocins have many characteristics such as high stability, low toxicity, and a narrow to a broad spectrum of activity, making them suitable for clinical applications as safe, natural food preservatives (Bharti et al., 2015; Kitagawa et al., 2019; Zacharof & Lovitt, 2012). Enterococcus, lactobacillus, pediococcus, leuconostoc, and carnobacterium are genera of LAB that commonly produce bacteriocins (Bharti et al., 2015). LAB such as lactobacillus sp., streptococcus sp., leuconostocsp., and Lactococcussp. isolated from dadih, a kind of fermented buffalo milk product, have been tested for their antimutagenic activity against different mutagens (Hosono et al., 1990a) and antimicrobial activity against listeria monocytogenes (Pato et al., 2020). The current work aimed to assess the antimicrobial activity of dadih’s LAB and to characterize the bacteriocin responsible for the antimicrobial activity against S. aureus.

2 Materials and methods

2.1 Media and chemicals

The media used for the activation of LAB and S. aureus FNCC-15 (Food and Nutrition Culture Collection) cultures and the antimicrobial test were MRS Broth (MRSB), Nutrient Broth (NB), and Nutrient Agar (NA). The chemicals used include ammonium sulfate, phosphate buffers, and chemicals for the molecular weight analysis of bacteriocin and amylase and proteolytic enzymes. All the media and chemicals were purchased from Merck and Sigma Aldrich (Singapore).

2.2 Pathogenic bacteria and Dadih’s LAB

The gram-positive pathogenic bacterium used was Staphylococcus aureus FNCC-15 was obtained from the Laboratory of Food Microbiology, Center for Food and Nutrition Studies, Universitas Gadjah Mada, Yogyakarta, Indonesia. Dadih samples were purchased from Bukittingi, West Sumatera, Indonesia. LAB was isolated and identified according to Hosono et al. (1990b) using API® 50 CH test.

2.3 Activation of the LAB culture and Staphylococcus aureus FNCC-15

The activation of LAB and S. aureus cultures was carried out according to the method described in our previous work (Pato et al., 2020). Each LAB culture was taken as much as 0.1 mL and put into a test tube containing 5 mL MRSB, then shaken evenly and incubated aerobically for 18 h at 37 °C. However, S. aureus was activated by inoculating 0.1 mL of the bacterial suspension into 5 mL NB, shaken evenly, and incubated aerobically for 18 h at 37 °C.

2.4 In vitro antimicrobial activity of LAB

LAB cultures were grown in MRSB and incubated aerobically for 24 h at 37°C; however, the indicator bacterium, S. aureus, was grown in NB for 24 h at 37 °C. One hundred µl of pathogenic microorganisms were placed and spread using glass hockey sticks on MRSA surface. The sterile disc papers were dipped into the LAB supernatants and sterile MRSB as a negative control. Disc papers with a diameter of 6 mm were then placed on the surface of the MRSA plates that been previously inoculated with indicator bacteria. The plates were then incubated at 37°C for 24 h, and the diameter of the growth inhibition zone was measured (Syukur et al., 2014a).

2.5 Production of bacteriocin

Dadih LABs were propagated in MRSB by adding inoculum as much as 10% of the active culture and incubation at 37 °C for 24 h. Then, the whole broth was centrifuged at 10,000 rpm for 15 min to get the supernatant. The supernatant separated from the cells was then added with 70% ammonium sulfate and put in the refrigerator (4 °C) for 12 h to precipitate the protein. This mixture was centrifuged at 10,000 rpm at 4 °C for 30 min to obtain crude bacteriocin (Sankar et al., 2012).

2.6 Bacteriocin characterization

Effect of pH

The effect of pH from 3 to 11 was carried out by adding 0.5 mL of bacteriocin into 4.5 mL of NB and then incubating for 30 min at 37 °C. After incubation, the antimicrobial activity of treated bacteriocin against S. aureus was assayed using the agar diffusion method (Syukur et al., 2014a).

Effect of temperature

The effect of heat treatment at 30 to 121 °C was carried out by adding 0.5 mL of bacteriocin into 4.5 mL of NB in test tubes. Each test tube was overlaid with paraffin oil and covered with aluminum foil to prevent evaporation during heating for 10 min at the temperature mentioned above. The antimicrobial activity of heated bacteriocins against S. aureus was carried out using the agar diffusion method (Syukur et al., 2014a).

Effect of amylase and proteolytic enzymes

The effect of amylase and proteolytic enzymes on the activity of bacteriocin was conducted according to the previous method (Zhou et al., 2014). The crude bacteriocin was treated with 5 mg/ml of amylase, trypsin, and protease, while phosphate buffer (0.5M, pH 7.0) was used as a control. The antimicrobial activity of the enzyme-treated bacteriocin against S. aureus was carried out using the agar diffusion method (Syukur et al., 2014b).
Purification and molecular weight determination of bacteriocin

The crude bacteriocin was dissolved in a phosphate buffer (0.1 M, pH 7.0) and dialyzed in the same buffer at 4 °C for 12 h. The dialyzed crude bacteriocin was then applied to the Sephadex LH-20 column (2.0 x 50 cm), which had been pre-filtered with the same phosphate buffer. The flow rate was set at 24 mL/h, and the fractions formed were collected as much as 10 ml each. The fractions that showed high antimicrobial activity were concentrated using a lyophilizer and then measured their molecular weight. The bacteriocin's molecular weight was determined using 12% SDS-PAGE gel electrophoresis in the LKB Bromma 2050 Midget electrophoresis unit (Pharmacia Amersham Co). The gel then was stained using Coomassie Brilliant Blue R-250 and destained by washing with a mixture of acetic acid-methyl alcohol-water (5:5:1 v/v) for 12 h. The low molecular marker (10-100 kDa) with six polypeptides was used as a marker (Rajaram et al., 2010).

Statistical analysis

The data obtained were analyzed using the SPSS 18.0 Program for Windows (Munich, Germany), including Analysis of Variance and Duncan New Multiple Range Test at the 5% level.

3 Results and discussion

A total of 12 LAB strains used in this study were isolated from dadih. The isolated LAB strains are Leu. Paramesenteroides R-8, St. cremoris R-14, St. faecalis subsp. Liquefaciens R-19, St. lactis subsp. Diacetylactis R-22, Leuconostoc para mesenteroides R-31, St. lactis subsp. diacetylactis R-41, St. lactis subsp. diacetylactis R-43, Leuconostoc para mesenteroides R-45, Leuconostoc para mesenteroides R-49, St. faecalis subsp. Liquefaciens R-55 and St. faecalis subsp. liquefaciens R-56. The inhibition zone of cell-free supernatant for the twelve strains of LAB against Staphylococcus aureus is shown in Figure 1. These results indicated the secretion of antibacterial compounds into the extracellular environment during LAB growth, as shown by the clear zone. The strains R-22, R-45, R-49, R-19 showed higher antimicrobial properties, while R-8, R-14, and R-31 showed lower antimicrobial properties. The difference in antimicrobial activity of the isolated 12 strains of LAB is likely due to differences in the amount of lactic acid or the type of bacteriocin produced by each LAB. These results are in agreement with the results reported by several investigators who reported that LABs can inhibit the growth of S. aureus (Syukur et al., 2014a, b; Othman et al., 2017). The current results also revealed that the strains R-8, R-14, and R-31 showed the lowest antimicrobial activity, so these three BAL strains were no longer used in the next tests.

The antimicrobial compounds released during LAB growth are generally in the form of organic acids, especially lactic acid, diacetyl, hydrogen peroxide, or proteinaceous bacteriocin (Bharti et al., 2015). To find out the antimicrobial compounds of the highest nine antimicrobial LABs, the tests were continued to estimate the sensitivity of their supernatant to various pH treatments, and the results are shown in Table 1. The supernatant of R-49 and R-56 lost their activity when the pH was adjusted from 7 to 11. However, the activity of R-22 was lost at pH 9 to 11. These results suggested that organic acids, especially lactic acid which produced during the growth of LAB, play a role in inhibiting S. aureus growth. Several researchers have previously reported similar results about antimicrobial activity of organic acids produced by various strains of LAB, mainly lactic acid, against S. aureus (Pato et al., 2017).

The antimicrobial components of the other six LAB strains, R-19, R-32, R-43, R-45, R-49, and R-55, are most likely proteinous bacteriocin. This is characterized by no loss in the inhibitory
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Table 1. Sensitivity of antimicrobial activity of supernatant from dadih’s lactic acid bacteria against Staphylococcus aureus at various pHs.

| Strains | Control | pH 3 | pH 5 | pH 7 | pH 9 | pH 11 |
|---------|---------|------|------|------|------|-------|
| R-19    | 11.77a | 9.97b | 7.73c | 14.30c | 11.07bc | 14.00d |
| R-22    | 2.77a  | 1.43b | 2.77a | 0.87c | 0*d  | 0*d  |
| R-32    | 3.77a  | 6.33b | 5.10b | 9.07b | 7.00b | 8.40d |
| R-41    | 7.87c  | 8.65c | 3.97b | 6.97bc | 4.10b | 8.30c |
| R-43    | 6.07c  | 7.60b | 7.10b | 11.00c | 5.87b | 6.63ab |
| R-47    | 7.67c  | 4.43b | 4.63b | 4.00b | 8.30c | 8.40b |
| R-49    | 3.43bc | 2.20b | 4.20b | 0*d  | 0*d  | 0*d  |
| R-55    | 9.37b  | 10.20c | 10.20c | 6.43c | 5.33c | 7.30b |
| R-56    | 3.00b  | 2.67b | 2.070b | 0*d  | 0*d  | 0*d  |

*a lost antimicrobial activity. Means followed by the lowercase letters in the same row indicate a significant difference (P < 0.05).

Table 2. Sensitivity of antimicrobial activity of crude bacteriocin from dadih’s lactic acid bacteria against Staphylococcus aureus at various heat temperatures.

| Strains | Control | 30 °C | 50 °C | 70 °C | 90 °C | 100 °C | 121 °C |
|---------|---------|-------|-------|-------|-------|--------|--------|
| R-19    | 6.42a  | 8.12b | 7.00d | 6.56e | 6.67c | 5.53c  | 2.00e  |
| R-32    | 2.77a  | 4.77a | 3.33b | 5.77c | 8.21d | 5.11b  | 0.67e  |
| R-41    | 5.43a  | 5.20a | 4.32b | 8.78c | 6.99c  | 7.32c  | 1.00e  |
| R-43    | 4.99a  | 5.87d | 4.20b | 6.55b | 6.76c | 4.10b  | 2.67c  |
| R-45    | 5.56a  | 5.35b | 3.77b | 6.32d | 5.53d | 6.77d  | 1.50c  |
| R-55    | 7.88a  | 5.89b | 7.22c | 7.47c | 7.88d | 7.53c  | 1.00e  |

Means followed by the lowercase letters in the same row indicate a significant difference (P < 0.05).

Table 3. Antimicrobial activity of crude bacteriocin from dadih’s lactic acid bacteria against Staphylococcus aureus treated with various enzymes.

| Strains | Inhibition zone (mm) |
|---------|---------------------|
|         | Control | Amylase | Trypsin | Proteinase |
| R-19    | 3.32a  | 13.46c | 0      | 0         |
| R-32    | 3.43a  | 6.80b  | 0      | 0         |
| R-41    | 3.53b  | 9.00b  | 0      | 0         |
| R-43    | 5.78b  | 9.66b  | 0      | 0         |
| R-45    | 7.66b  | 7.23b  | 0      | 0         |
| R-55    | 7.43c  | 4.51b  | 0      | 0         |

*a lost antimicrobial activity. Means followed by the lowercase letters in the same column indicate a significant difference (P < 0.05).

Additionally, the data in Table 2 also showed that the compounds that are probable to be bacteriocin presented in the supernatants were still resistant to temperatures up to 121 °C, even though there was a significant decrease in their antimicrobial activity (P < 0.05). Cell-free supernatant from the six dadih’s LAB is shown to induce antimicrobial activity, resistant to various heating temperatures and pH. The sensitivity of the produced bacteriocin was further tested using various enzymes, and the results are presented in Table 3. These results indicated that treatment with amylase resulted in the loss of antimicrobial activity of crude bacteriocin against S. aureus and suggested that the component of the crude bacteriocin responsible for the antimicrobial effect is not starch but protein. This statement was supported by the loss of antimicrobial activity of crude bacteriocin treated with proteolytic enzymes such as trypsin and proteinase K. Thus, the current results showed that the bacteriocin produced by LAB is a highly thermostable proteinaceous compound with antimicrobial activity even after autoclaving at 121 °C (Perez et al., 2014). Most of the LAB bacteriocins are peptide compounds, heat resistant, cationic, and amphiphilic (Zacharof & Lovitt, 2012).

The molecular weight of bacteriocins isolated and purified from St. faecalis subsp. liquefaciens R-55 is presented in Figure 3.
Based on SDS-PAGE analysis, it is found that the molecular weight of this bacteriocin was 14.4 kDa. The bacteriocins produced by several LAB vary to a great extent depending on the genus and species. Some bacteriocins have a molecular weight of greater, less, or almost the same as the molecular weight of bacteriocins produced by the R-55 strain. The molecular weight of bacteriocins from Lactobacillus plantarum isolated from Chinese pikel was almost the same as the bacteriocin molecular weight produced by strain R-55, namely 16.5 kDa (Zhou et al., 2014). However, Lactobacillus lactis isolated from the marine environment produced bacteriocin with a molecular weight of 94 kDa, much greater than that produced by R-55. Most LAB bacteriocins are peptide compounds with small molecular weights of less than 10 kDa (Zacharof & Lovitt, 2012). Enterococcus faecalis KT11 produced bacteriocin with a molecular weight of 3.5 kDa (Abanoz & Kunduhoglu, 2018), and Lactobacillus viridescence NICM 2167 released bacteriocin with a molecular weight of 8.3 kDa (Sure et al., 2016). The bacteriocin produced by Pediococcus pentosaceus zy B isolated from the intestine of Mimachlamys nobilis had a molecular weight of 2.22 kDa (Zhang et al., 2020). Plantaricin produced from Lactobacillus plantarum IIA-1A5 showed a size of about 9.6 kDa (Fatmarani et al., 2018).

4 Conclusions

The current study showed that 12 LABs were isolated from dadih, and all of them could suppress the growth of S. aureus with various inhibition zone. The antimicrobial activity of LAB against S. aureus is mainly due to the production of organic acids, especially lactic acid by strains R-8, R-14, and R-49 or the production of bacteriocins by strains R-19, R-32, R-41, R-43, R-45, and R-55. The bacteriocin derived from R-55 showed the highest antimicrobial activity against S. aureus among the bacteriocin-producing LAB. The molecular weight of bacteriocin produced by R-55 was 14.4 kDa. This study concluded that dadih is a suitable source for the production of LAB, which can produce the antimicrobial bacteriocin to be used in the preservation of food against the pathogenic S. aureus.

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Figure 3. SDS-PAGE analyses for molecular weight of bacteriocin produced by St. faecalis subsp. liquefaciens R-55.
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