The antibacterial activities of bacteriocin *Pediococcus acidilactici* of breast milk isolate to against methicillin-resistant *Staphylococcus aureus*

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Abstract. Resistance has occurred if an antibiotic cannot kill pathogenic bacteria therefore an antibiotic therapeutic effect cannot be achieved. Broad and free use of antibiotics effected in health problems, one of the health problems is bacteria are experienced the resistance such as Methicillin-Resistant Staphylococcus aureus (MRSA). Therefore an antibiotic alternative is needed from natural ingredients have the potential as anti-MRSA such as bacteriocin. This research was conducted to determine the antibacterial activity of bacteriocin from *Pediococcus acidilactici* of breast milk isolate (ASI) against MRSA. Bacteriocin isolation was carried out by growing of *P. acidilactici* on the media of Rogosa Sharpe (MRS) broth. Antibacterial activity test was carried out by diffusion method are to see the inhibition zone and dilution to determine the value of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC). The test results shown that bacteriocin *P. acidilactici* strains A11 and C12 strains had antibacterial activity against MRSA. The A11 strain inhibition zone is 15 mm, MIC and MBC values are 25%. While the C12 strain inhibition zone produced 18 mm, the value of MIC and MBC was 12.5%.

1. Introduction

Extensive and inappropriate use of antibiotics has caused bacteria to experience resistance. Bacterial resistance is a condition where the bacteria can survive or not die when attacked by antibiotics, even have the ability to adapt and develop when exposed to antibiotics [1]. According to the CDC U.S, The Department of Health and Human Services in 2013, 23,000 people has died each year from disease infections accompanied by antibiotic resistance. Resistance events such as the discovery of Methicillin-resistant *Staphylococcus aureus* (MRSA) in acute infections are a public health threat. MRSA is a strain of *Staphylococcus aureus* that is resistant to the methicillin group. The high rate of bacterial resistance encourages efforts to utilize natural ingredients that have antibacterial activity which is used as an antibiotic agent. One natural product such as bacteriocin lactic acid bacteria (LAB) has the potential as an antibacterial in the pharmaceutical field [2].
BAL is a group of bacteria have a Gram positive, not have spores, do not have cytochrome, aerotolerant, anaerobic, have microaerophilic, spherical or stem which produces lactic acid as the main metabolic end product during carbohydrate fermentation, H$_2$O$_2$ anticancer and antimicrobial in bacteriocin [3]. Bacteriocin is an extracellular enzyme as a probiotic are contributes to suppressing pathogenic bacteria in the digestive tract [4]. The advantage of this compound compared to other antibacterial compounds is works selectively, is safe and is able to prevent resistance [5]. Bacteria is can produce bacteriocin are LAB groups, one of which is isolated from Breast Milk.

Breast Milk is a source of nutrition for babies containing LAB [6]. Breast milk contains bifidogenic factors, an oligosaccharide called acetylglucosamine and glycoprotein which can support the growth of LAB especially bifidobacteria [7]. Previous study was able to isolate probiotic bacteria from breast milk is namely the genus Lactobacillus which could potentially reduce cholesterol [8]. This study aims to determine the antibacterial activity of LAB bacteriocin isolates breast milk against MRSA.

2. Research methods
This study uses a type of experimental test research with the well diffusion method. The research object is bacteriocin obtained from LAB breast milk isolates. MRSA bacterial samples were obtained from the research sites of the Microbiology Laboratory of the Faculty of Nursing and Health Sciences of the University of Muhammadiyah Semarang. MRSA bacteria strain from diabetic ulcer base swabs and resistant to antibiotics Methicillin, Benzylpenicillin, Oxacillin, Gentamicin, Ciprofloxacin, Levofloxacin, Moxifloxacin, Trimethoprim/Sulfamethoxazole.

2.1. Tools and materials
The tools has used are autoclave (Huramaya), incubator (Memmert), centrifuge (Gemmy), refrigerator (Frimed), freezer (Electrolux), laminar air flow (Mascotte), petri dish (disposible), micropipette. The ingredients used are breast milk, media by Roger Rogosa Sharpe (MRS) broth, Man of Rogosa Sharpe (MRS) so that the media Muller Himton Agar (MHA), physiological NaCl, bacteria test MRSA, aquadest, 70% alcohol, St. Mc. Farland, and antibiotics (Amphicilin, Eritromycin, Gentamycin, Tetracycline).

2.2. Isolation of Bacteriocin
Bacteriocin was isolated from the LAB of Pedioococcus acidilactici strains A11 and C12 which were then cultured again in the media DeMan, Rogosa, Sharpe (MRS) broth, and would produce a large amount of LAB which was characterized by turbidity. After being cultured on the media then centrifugation in cold conditions to get the bacteriocin content in BAL [9]. Next part of the supernatant was taken and 1N of NaOH was added and therefore bacteriocin was obtained in a neutral state.

2.3. Bacterial Inoculation
MRSA are available at the Microbiology Laboratory of the Faculty of Nursing and Health Sciences of the University of Muhammadiyah Semarang was inoculated into the media of Blood Plate Agar (BAP) at a temperature of 35 ± 2 ºC for 24 hours. And the next step, the colony with positive catalase was selected and continued with the Fire Staph test. Furthermore, sensitivity was tested for antibiotics gentamicin, ciprofloxacin, levofloxacin, clindamycin, tetracycline, rifampicin and trimethoprim.

2.4. Antibacterial Activity Test
Antibacterial activity test was carried out by the well diffusion method. The test bacteria were subcultured on BAP media for 24 hours at a temperature of 35 ± 2°C. Then turbidity 0.5 of the McFarland standard (1.5 × 108) is made. Using sterile cotton is blocked throughout the MHA media. Then has made wells by using a cork borer with a diameter of 0.5 cm. The antibacterial activity of bacteriocin was carried out by bacteriocin isolated from 200 µL to each well. Then it incubated at 35 ± 2oC for 16-20 hours. Antibacterial activity is determined by measuring the inhibitory zone [10].
2.5. **Minimum inhibitory concentration (MIC)**

The antibacterial activity of bacteriocin was carried out by microplate using microplate. The method of microdilution is a method recommended by the Clinical and Laboratory Standards Institute (CLSI). Microplate consists of 96 wells, 12 columns and 8 rows. The wells used are 24 wells, each well filled with 100 µL MHB. The first column was added 100 µL bacteriocin strain A11, while the second column was added 100 µL bacteriocin strain C12. Then each bacteriocin is resuspended to the next column until column 7. Then each column is filled with a suspension of the MRSA test with turbidity of 0.5 McFarland standard (1.5 × 108) as much as 100 µL. Microplate is incubated at 35 ± 2°C for 16-20 hours. MIC is the smallest concentration where there is no bacterial growth in the well which is shown in clear color and carried out by visual observation.

2.6. **Minimum bactericidal concentration (MBC)**

MBC is done by taking a solution from the microplate well which shows the MIC value and from all other wells that are above the MIC value using the ose. Then it was scratched on the surface of the prepared BAP media. Then BAP was incubated at 35 ± 2°C for 16-20 hours. BAP which shows the visualization of clarity and non-bacterial overgrowth was determined as the MBC value.

3. **Result and Discussion**

3.1. **Regeneration of Pediococcus acidilactici**

The bacteriocin-producing *P. acidilactici* isolate used must always be regenerated so that the bacteria are in the growth phase. Bacterial rejuvenation is an important thing that must be done to get new bacterial cultures so that they can reproduce well when used [11]. According to [12], regeneration of *P. acidilactici* using MRSA media for 48 hours to provide nutrition and grow bacteria, at the 48th hour *P. acidilactici* was ready to be harvested and inoculated into the MRSB media to make bacteriocin.

![Figure 1](image1.png)

**Figure 1.** Isolates of *Pediococcus acidilactici* strains C12 (A) and A11 (B).

3.2. **Bacteriocin Production**

Bacteriocin isolation was made by inoculating 2 groups of *P. acidilactici* isolates into 200 mL MRSB media for 48 hours at 37°C. Then centrifugation at a speed of 12000 rpm at 4°C for 10 minutes. Then the part of the supernatant is separated and add NaOH 1 N to get bacteriocin in neutral.

![Figure 2](image2.png)

**Figure 2.** Bacteriocin.
### 3.3. Antibacterial Activity of Bacteriocin

The antibacterial activity of bacteriocin in the *P. acidilactici* strains A11 and C12 was seen with the resulting inhibition zone. Testing is done with three repetitions. The inhibitory zone that is formed is measured using a caliper.

**Table 1.** Average Diameter of Bacteriocin Inhibitory Zone.

| Bacteria | Inhibition zone of bacteriocin (mm) |
|----------|-----------------------------------|
|          | A11 | C12 |
| MRSA     | 15  | 18  |

In Table 1, shows that bacteriocin is able to inhibit the growth of MRSA in the presence of the resulting clear zone of 15 mm and 18 mm. According to [13], the diameter of the inhibition zone with a value of 0-3 mm is included in the weak category, the inhibition zone between 3-6 mm is included in the medium category and the inhibition zone is more than 6 mm in the strong category. So it can be concluded that bacteriocin antibacterial activity of *P. acidilactici* ASI isolates against MRSA is included in the strong situation. These results further confirm that bacteriocin is a safer antibacterial agent and prevents resistance.

**Table 2.** Differences between MRSA treatment with bacteriocin and antibiotics.

| No | Treatment of MRSA | Antibiotics (mm) | Bacteriocin (mm) |
|----|--------------------|------------------|------------------|
| 1  | Amphicilin (0)     | Eritromycin (0)  | A11P (15)        |
|    |                    | Genatamycin (0)  |                  |
|    |                    | Tetracycline (18)|                  |
| 2  | Amphicilin (0)     | Eritromycin (0)  | C12P (18)        |
|    |                    | Genatamycin (0)  |                  |
|    |                    | Tetracycline (18)|                  |

As for bacteriocin testing and antibiotics control of MRSA in Table 2, the results of the Kriskal Wallis test showed a significance value of 0.735 (p ≥ 0.05). This means that there is no difference between the treatment of MRSA and bacteriocin and antibiotics.
Table 3. The bacteriocin MIC values for MRSA.

| Bacteriocin      | MIC | 100% | 50% | 25% | 12.5% | 6.25% | 3.12% | 1.5% |
|------------------|-----|------|-----|-----|-------|-------|-------|------|
| P. acidilactici A11 | -   | -    | -   | +   | +     | +     | +     | +    |
| P. acidilactici C12 | -   | -    | -   | -   | +     | +     | +     | +    |

Figure 4. The bacteriocin MIC values of A11 (5) dan C12 (6) against MRSA.

Table 4. MBC bacteriocin values against MRSA.

| Bacteriocin      | MBC | 100% | 50% | 25% | 12.5% | 6.25% | 3.12% | 1.5% |
|------------------|-----|------|-----|-----|-------|-------|-------|------|
| P. acidilactici A11 | -   | -    | -   | +   | +     | +     | +     | +    |
| P. acidilactici C12 | -   | -    | -   | -   | +     | +     | +     | +    |

Figure 5. MBC value of bacteriocin A11 (E) dan C12 (F) against MRSA.

The results of testing the determination of MIC and MBC from each bacteriocin against MRSA can be seen in Table 2. and Table 3. The MIC and MBC values of bacteriocin A11 and C12 were respectively 25% and 12.5%. The MBC value will always be higher or equal to the MIC value.

The target of bacteriocin from LAB is the sensitive cytoplasmic membrane of bacterial cells because the initial bacteriocin reaction is to damage membrane permeability and eliminate proton motive force (PMF) thereby inhibiting energy production and biosynthesis of proteins or nucleic acids. Bacteriocin inhibition activities require cell surface specific receptors, for example in pediocin AcH. Besides that it results in cell lysis. This is a secondary effect of pediocin AcH activity through depolymerization of the peptidoglycan layer, so that it can indirectly activate the cell autolysis system [14].

The mechanism of bacteriocin bactericidal activity is as follows: (1) bacteriocin molecules are in direct contact with cell membranes, this contact process is able to disrupt membrane potential in the destability of cytoplasmic membranes so that cells become not strong, and (3) membrane instability capable of producing holes cell membrane through the process of interference with PMF (Proton Motive Force). Leaks that occur due to the formation of holes in the cytoplasmic membrane are indicated by the presence and exit of cellular molecules. This leakage results in a decrease in cellular pH gradient. The effect of forming cytoplasmic holes is the impact of bacteriocin which causes changes in gradient potensal membrane and the release of intracellular molecules as well as the entry
of extracellular substances (environment). The effect causes cell growth to be inhibited and produce a death process in cells that are sensitive to bacteriocin [15].

Bacteriocin as a bacterial metabolite has the potential to be utilized in therapeutic effects. But there are fundamental differences between bacteriocin and antibiotics. Bacteriocin is generally a peptide or peptide complex with intraspecific antagonistic effects on the strain of the product and relatively narrow bactericidal spectrum, this is what distinguishes it from antibiotics.

One of the advantages of using bacteriocin LAB as an antimicrobial is its ability to eliminate pathogenic microbes and decompose food from milk and meat. The possibility of further therapeutic applications is closely related to the characteristics of the antimicrobial substance, which is not toxic, can inhibit low levels and is produced by bacteria classified as GRAS (generally recognized as safe), which is a microbe that is not at risk to health. Thus, it provides an opportunity for its use as an antimicrobial agent against MRSA.

4. Conclusion
The test results can be concluded that the two bacteriocin strains of *P. acidilactici* has shown potential antimicrobial activity against MRSA with inhibitory zone values of 15 mm and 18 mm, respectively. While the value of MIC and MBC are 25% and 12.5% respectively. So it is confirmed that bacteriocin *P. acidilactici* isolates ASI has the potential as a natural antibacterial agent that can prevent resistance and is not toxic. The advantages of using bacteriocin, especially from LAB as an antimicrobial substance, are able to inhibit low levels and are produced by bacteria classified as GRAS (generally recognized as safe), which is a microbe that is not at risk to health. Thus, it provides an opportunity for its use as an antimicrobial agent against MRSA.

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