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Bacteriocin Activity of Lactic Acid Bacteria Isolated from Rumen Fluid of Thin Tail Sheep

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ABSTRACT

The objective of this study was to determine the activity and the stability of bacteriocin from lactic acid bacteria (BAL) isolated from rumen fluid of thin-tail sheep under the temperature (80, 100, and 121°C), pH (3, 7, and 10), and the length of storage (for 2 weeks under the temperature -8, 11, and 29°C). Lactic acid bacteria obtained by isolation, selection, and identification of thin-tailed sheep rumen fluid were used for bacteriocin production. The crude bacteriocin was partially purified using 70% ammonium sulfate, then was dialysis for 12 hours. The obtained bacteriocin then tested its inhibitory activity against *E.coli* (representing Gram-negative) and *S. aureus* (representing Gram-positive) under temperature (80, 100, and 121°C), pH (3, 7, and 10), and the length of storage (for 2 weeks under the temperature -8, 11, and 29°C). The data of bacteriocin activity based on pH, temperature, and the length of storage were analyzed with factorial, then when there was a significant difference of variable because treatment was continued with Duncan's Multiple Range Test (DMRT) test. The results showed that the bacteriocin activity of the three types of BAL against *S.aureus* is greater than *E.coli*. The highest activity was shown in pH 3, while the lowest activity was shown at pH 10 ($P<0.01$). The highest activity was shown at a heating temperature of 100°C, while the lowest activity was shown at a heating temperature of 80°C ($P<0.01$). The activity of bacteriocin produced by BAL 0 A, BAL 1 A, and BAL 4 C tended to be stable to the heating temperature of 80, 100, and 121°C but decreased with increasing pH value (pH 3, 7, and 10). The best of bacteriocin activity was found at pH 3 (acid), heating at 100°C, and stored at -8°C for 14 days.

Keywords: Bacteriocin activity, *Escherichia coli*, Lactic acid bacteria, *Staphylococcus aureus*

Introduction

Uncontrolled use of antibiotics can lead to the emergence of certain bacterial resistance to livestock and leave antibiotics residual on livestock products that can threaten public health. The wide scope of antibiotics work can cause all types of bacteria found in the gastrointestinal tract to be killed, including beneficial bacteria for livestock. These problems form the basis of reducing antibiotic use in efforts to increase livestock production.

Reduced antibiotic use in livestock can be achieved if alternative antimicrobial available. Bacteriocin is defined as an antimicrobial compound in the form of a protein that is synthesized by various species of bacteria including a group of lactic acid bacteria (LAB) in the ribosome. Some bacteriocins are produced from lactic acid bacteria that are associated with fermented products (Galvez *et al*., 2007). The benefits of bacteriocin as a bio preservative include: (1) it is non toxic and easily degraded by proteolytic enzymes; (2) harmless to the intestinal microflora because it easily digested by digestive enzymes; (3) reduce the use of harmful preservative chemicals; (4) flexible to use; (5) more stable over a wide range of pH and temperature, tolerant of processes using acids or bases, as well as hot and cold conditions (Cleveland *et al*., 2001). Based on these, bacteriocin is one of the compounds that have the potential as an alternative to antibiotics both in animal and humans in the context of preserving feed or food ingredients and killing pathogenic bacteria in the digestive tract.

Indonesia is a country with the tropical and humid climate, so it has biodiversity including a high diversity of microorganisms and many microorganisms are found, especially bacteria that have the potential to produce bacteriocin in various hosts and their habitats. Rumen fluid is

one of the places where microorganisms are found such as protozoa, bacteria, and fungi. Lactic acid bacteria (LAB) are found in the digestive tract, especially in the rumen (Stewart, 1992). Some strains of lactic acid bacteria produce bacteriocin which has bactericidal activity on Gram-positive and Gram-negative bacteria (Tahara *et al*., 1996).

Factors that influence the stability of bacteriocin include environmental conditions such as temperature and pH (Usmiati and Rahayu, 2011). Bacteriocin also showed stable activity after being treated at a temperature of -20ºC to 100ºC (Abubakar dan Arpah, 2015). Stability of liquid bacteriocin extract from Lactobacillus sp. SCG 1223 strains against *E.coli* decreased after 4 months of storage at pH>7 (Usmiati and Noor, 2009). Thin-tail sheep are one of the local ruminant animals that can be used as a source of rumen fluid as a good source of lactic acid bacteria. This is the basis for isolation and selection of lactic acid bacteria (LAB), which has the potential to produce bacteriocin from thin-tail sheep rumen fluid. Selected lactic acid bacteria are used for bacteriocin production. Bacteriocin is a protein compound, factors that affect protein stability can also affect the stability of bacteriocin. The bacteriocin obtained was then tested for its inhibitory activity against pathogenic bacteria *E. coli* (Gram-negative) and *S. aureus* (Gram-positive) under the treatment of pH, temperature, and duration of storage, so that it can be seen the stability of bacteriocin which can later be applied to bio preservative agents which safer for livestock and food.

Materials and Methods

Materials

This research was conducted in May 2017 to February 2018 in the Laboratory of Nutrition Biochemistry, Faculty of Animal Husbandry, Universitas Gadjah Mada. The tools used are equipment for culturing bacteria, autoclaves, magnetic stirrers, bunsen lamp, Hungate tubes, Erlenmeyer, beaker glass, spectrophotometers, laminar airflow, analytical scales, Petri dish, pH-meter, safe lock tubes, microscopes, micropipettes, calipers, centrifuge, plastic, and incubator.

Bacteria used were lactic acid bacteria from isolation, selection and characterization of thin-tail sheep rumen fluid (BAL 0 A, BAL 1 A, and BAL 4 C). The thin-tail sheep used were weaning off sheep treated with lactic acid bacteria based fermented feed. The medium used is MRS broth, nutrient broth, and agar for lactic acid bacteria growth. Other materials are aquadest and reagents for testing the stability of inhibition against *E. coli* and *S. aureus* bacteria.

Methods

This research was divided into 3 steps. The first step was the culturing of lactic acid bacteria. The second step was bacteriocin production and partial purification. The third step was the bacteriocin stability test.

Step I. Culturing of lactic acid bacteria

This research used 3 types of lactic acid bacteria isolated from thin-tail sheep rumen fluid, that was BAL 0 A, BAL 1 A, and BAL 4 C. Each isolate was enriched in MRS broth medium. Each lactic acid bacteria was enriched in 2 Hungate tubes. Lactic acid bacteria were incubated in an incubator for 24 hours at 37°C.

Step II. Bacteriocin production and partial purification

Bacteriocin production. Production of crude bacteriocin refers to Ogunbanwo *et al*. (2003). Isolates that have been selected and exhibit antimicrobial activity were enriched on CM sucrose at 37°C for 48 hours. Cell culture was centrifuged (8000 rpm, temperature 4°C for 15 minutes) to get the supernatant. The cell-free supernatant is adjusted to pH 6 by adding 1 M NaOH (with the intent to eliminate the effect of organic acids), then filtered with 0,2 µm cellulose acetate filter. Furthermore, the cell-free supernatant was heated at 80°C for 10 minutes to eliminate proteolytic activity and hydrogen peroxide. Cell-free supernatant is a crude bacteriocin.

Bacteriocin purification. Partial purification of the sample was carried out by precipitation using $(NH_4)_2SO_4$ 70%, then continued by dialysis process for 12 hours. The dialysis bacteriocin extract was collected and centrifugated at 10000×g at 4°C for 15 minutes. The extract was dissolved using Phosphate buffer (0.1 M, pH=7.0) and stored at 4°C for further use (Sharma *et al*., 2011).

Step III. Stability bacteriocin test

Bacteriocin activity test. The bacteriocin activity test used agar well diffusion method (Ogunbanwo *et al*., 2003; Udhayashree *et al*., 2012). 100 µl indicator bacteria were inoculated on the MHA media, then a 6 mm diameter well was made on agar media. In each well, 10 µl of crude bacteriocin was added. The inoculant was incubated at 35°C for 24 hours. Bacteriocin activity is indicated by the presence of a clear zone around the well. Bacteriocin activity is expressed as Arbitration Unit (AU) per ml. One AU is expressed as the inhibitory zone area per unit volume of the bacteriocin sample tested (mm^2/ml) (Usmiati and Marwati, 2007).

$$\text{Bacteriocin activity } (mm^2/ml) = AU/ml = \frac{Lz - Ls}{V}$$

Note:
Lz = clear zone area (mm^2)
Ls = well area (mm^2)
V = sample volume (mL)

Stability of bacteriocin to pH. A total of 5 ml of crude bacteriocin in different tubes, each pH adjusted pH 3, 7, and 10 using the solution of NaOH or HCl. Bacteriocin activity was tested using the diffusion method in order that after incubation for 4 hours at room temperature.

Stability of bacteriocin to temperature. The bacteriocin was taken 200 µl, then was tested for stability to temperature for 80ºC for 20 minutes, 100ºC for 20 minutes and 121ºC for 15 minutes (Osmanagaoglu *et al*., 1998; Mandal *et al*., 2008).

Stability of bacteriocin during storage. The stability test during storage is carried out by storing bacteriocin at -8°C, 11°C, and 29°C for 2 weeks. The next step is the activity test using the diffuse agar method (Nugroho and Rahayu, 2003).

Data analysis

Data of bacteriocin resistance to pH, temperature, and duration of storage were analyzed using the factorial method, then if there were significant differences because the treatment was continued with Duncan's Multiple Range Test (DMRT) (Steel dan Torrie, 1995).

Result and Discussion

Bacteriocin activity

Bacteriocin activity is shown by measuring the inhibition of bacteriocin against pathogenic bacteria. The pathogenic bacteria used are *E.coli* (Gram-negative) and *S.aureus* (Gram-positive). The bacteriocin inhibitory activity of selected lactic acid bacteria is presented in Table 1.

Based on the result in Table 1, the bacteriocin activity of the three types of lactic acid bacteria (LAB) showed very significantly different results (P<0.01), as well as the bacteriocin activity against Gram-positive and negative pathogenic bacteria (P<0.01). The highest bacteriocin activity against pathogenic both *E. coli* and *S. aureus* was found in BAL 0 A, while the lowest activity was found in BAL 1 A. All three types of lactic acid bacteria had significantly greater bacteriocin activity (P<0.01) against *S. aureus* (Gram-positive) compared to *E.coli* (Gram-negative). Te activity of lactic acid bacteria against Gram-positive bacteria is greater than Gram-negative bacteria because lactic acid bacteria are Gram-positive bacteria, so the resulting bacteriocin has active properties against other species that have close relationships with producing microorganism, such as *S.aureus* which is a Gram-positive bacteria. However, the bacteriocin produced from the three types of lactic acid bacteria showed the ability to fight both types of pathogens both Gram-positive and Gram-negative. Research by Usmiati and Marwati (2007) explained that bacteriocin from *Lactobacillus* sp. Gallus SCG 1223 showed a large spectrum of inhibition zones against Gram-positive (*Listeria monocytogenes*) and Gram-negative (*Salmonella typhimurium, Escherichia coli*). Lactic acid bacteria tended to be active against various types of bacteria that are closely related to Gram-positive bacteria (Jack *et al.,* 1995). Gram-negative bacteria are generally insensitive to bacteriocins from the strain of lactic acid bacteria because the outer membrane of Gram-negative bacteria is equipped with a barrier wall, which is composed of lipopolysaccharides, lipoproteins, and phospholipids. The sensitivity of Gram-negative bacteria can be increased by injuring the cell (*sublethal injury*) using high hydrostatic pressure and utilizing the electric field as a non-thermal method. The activity of bacteriocin against Gram-negative was seen when the integrity of the bacterial outer membrane was disturbed for example by osmotic pressure, low pH, the presence of detergent, chelating agents, electric vibrations, and high pressure (Caplice and Fitzgerald, 1999; Stevens *et al.,* 1991; De Vuyst and Leroy, 2007).

Kusmarwati *et al*. (2014) explained that the crude bacteriocin extract produced by *Pediococcus pentosaceus* (Gram-positive) produced the highest antibacterial activity against *Staphylococcus aureus* (Gram-positive). Klaenhammer (1988) stated that bacteriocin is a protein compound that showed antibacterial activity (bactericidal or bacteriostatic) against sensitive species of bacteria. These protein are often active against other species that have close relationships with the producing bacteria. Other studies explained that bacteriocin derived from Gram-positive lactic acid bacteria (*L. plantarum* ST16Pa) can inhibit Gram-negative bacterial pathogens such as *E. coli* (Todorov *et al.,* 2011). The ability of antibacterial action of bacteria depended on several factors such as the concentration and purity during the bacteriocin preparation process, the type of buffer used, the sensitivity of the indicator of strain being tested, and the density of the cell suspension used (Leroy and De Vuyst, 2000).

Stability of bacteriocin to pH

Crude bacteriocin that has been produced was tested for its resistance to various pH values, including acidic conditions (pH 3), neutral (pH 7), and bases (pH 10). The stability of the bacteriocin activity treated with pH is presented in Table 2.

Based on Table 2, the pH treatment influences the bacteriocin activity of three types of lactic acid bacteria significantly (P<0.01). The higher pH treatment caused bacteriocin activity to decrease (P<0.01). This can be seen from the average activity of each bacterium against the given pH treatment. The highest activity is shown in pH 3, while the lowest activity is shown in the pH 10. In pH 3, the ability of pathogenic bacteria to grow will be reduced or even inhibited, compared to pH 10. Bacteriocin is a protein compound, whereas protein has a side that was sensitive to pH because it can change the structure of its protein constituents. Acid and base conditions can affect the degree of ionization of the protein so that when it reaches an isolated point, the protein can be denaturated. This condition can disturb the function of the protein. The loss of bacteriocin at pH 10 was caused by

Table 1. Inhibitory activity of bacteriocin from selected lactic acid bacteria

LAB Strain	Inhibitory activity (AU/ml)		Mean ± SD
	E. coli	*S. aureus*	
BAL 0 A	2,760.37±323.07	2,974.16±118.50	2,867.26±247.14[a]
BAL 1 A	1,592.35±38.43	2,647.07±213.89	2,119.71±593.82[c]
BAL 4 C	2,148.02±367.94	2,885.82±131.88	2,516.92±473.72[b]
Mean ± SD	2,166.92±562.42[x]	2,835.68 ± 201.91[y]	

[a,b,c] Different superscripts in the same column show very significant differences (P<0.01)
[x,y] Different superscripts on the same line show very significant differences (P<0.01).

Table 2. Bacteriocin stability of selected lactic acid bacteria to pH

LAB strain	pathogen	Inhibitory activity (AU/ml)			Mean ± SD
		pH 3	pH 7	pH 10	
BAL	*E. coli*	7,599.48± 233.78	7,766.32±18.76	3,883.51±21.87	
0 A	*S. aureus*	4,104.56±395.51	2,582.49±479.15	2,051.88±232.48	
	Mean	5,852.02±1,936.18	5,174.41±2,855.45	2,976.70±1,014.04	4,664.71±2,325.68[a]
BAL	*E. coli*	8,411.59±223.22	4,309.18± 215.10	3,145.13±177.23	
1 A	*S. aureus*	5,081.83±338.18	3,202.17±102.39	2,601.54±444.34	
	Mean	6,746.71±1,841.70	3,755.67±624.77	2,873.33±424.48	4,458.57±2,018.55[b]
BAL	*E. coli*	7,141.62±666.46	2,855.36±108.89	2,322.34±148.26	
4 C	*S. aureus*	4,592.36±480.06	4,406.38±25.88	1,801.48±194.32	
	Mean	5,866.98±1,489.79	3,630.87±852.47	2,061.91±324.47	3,853.25±1,865.13[b]
Mean ± SD		6,155.24±1,714.13[x]	4,186.98±1,801.55[y]	2,634.32±749.28[z]	

[a,b] Different superscripts in the same column show very significant differences (P<0.01)
[x,y,z] Different superscripts on the same line show very significant differences (P<0.01)

degradation by proteolytic enzymes, protein aggregation, re-absorption by producing cells, and regulation of feedback (Todorov and Dicks, 2006). Bacteriocin degradation occurs along with the producing cell death phase. Initially, the cell undergoes lysis then is followed by the accumulation of intracellular proteases that cause bacteriocin damage (Avonts *et al.,* 2004).

Bacteriocin derived from the type of BAL 0 A had the highest highly significant activity (P<0.01) compared to bacteriocin from BAL 1 A and BAL 4 C. Bacteriocin activity between BAL 1 A and BAL 4 C showed no difference. Based on the identification, BAL 0 A were identified as *Lactobacillus brevis*, while BAL 1 A was identified as a mixture of *Lactobacillus rhamnosus, Lactobacillus plantarum, Lactobacillus pentosus*, and BAL 4 C was identified as a mixture of *Lactobacillus brevis, Lactobacillus plantarum,* and *Pediococcus pentosus*. Based on the identification, each strain can produce different types of bacteriocin that had a different activity to inhibit other pathogen bacteria. Research by Ganzle *et al.* (1999) showed that the effect of pH treatment in the range of 4,9-6,5 affected bacteriocin activity against *L.innocua, E.coli* LTH1600, *E.coli* LTH4346, and Heidelberg type *Salmonella*. Increased sensitivity of nisin, sakacin P, and curvacin A to target bacteria occurs at low pH. Likewise in the study of Ogunbanwo *et al.* (2003), stated that the highest antibacterial activity of bacteriocin produced by *L. brevis* and *L. plantarum* was shown at acidic pH in the range of 2-6. Sankar *et al.* (2012) stated that the bacteriocin produced by *L. plantarum* isolated from cow's milk had good activity between pH 3 to 11 (has a high pH range). Messens and De Vuyst (2002) explained that many bacteriocin exhibit greater anti-bacterial activity at low pH (pH 5 and below) than their physiological pH because more bacteriocin molecules are produced at low pH. Low pH conditions cause increased solubility of bacteriocin, a reduction in hydrophobic peptide aggregation, and a reduction in bacteriocin binding on the cell surface. Hydrophilic bacteriocin has an increased capacity to pass through the hydrophilic region of the cell surface of sensitive target bacteria (Jack *et al.,* 1995).

Stability of bacteriocin to temperature

Stability of bacteriocin activity against Gram-positive and Gram-negative there are several factors that must be considered such as pH and incubation temperature. When the temperature used is in accordance with the conditions of microbes, the microbes that are cultured will grow optimally, and can produce optimal bacteriocin as well as the pH of the medium used to enrich the microbes. Therefore, it is necessary to test the stability of bacteriocin activity under temperature treatment, so it can be known the range of temperature that does not inhibit bacteriocin activity. The stability of bacteriocin activity against temperature is presented in Table 3.

Based on Table 3, the bacteriocin activity was still stable at 80°C, while at 100°C and 121°C the bacteriocin activity decreased. Based on total bacteriocin activity against the pathogen *E.coli* and *S.aureus*, the highest activity was found in bacteriocin produced by BAL 0 A. The results of bacteriocin activity from BAL 1 A did not differ greatly from BAL 0 A and BAL 4 C, while the bacteriocin activity from BAL 4 C is different from BAL 0 A which is lower. The mechanism of bacteriocin resistance to heat is related to the molecular structure of bacteriocin which is a simple peptide without tertiary structure (Usmiati and Rahayu, 2011).

The stability of the bacteriocin to temperature depends on the structure of the bacteriocin and the type of bacteriocin produced. Some types of bacteriocin are resistant to high temperatures, but some are also susceptible to

Table 3. Bacteriocin stability of selected lactic acid bacteria to temperature

LAB Strain	Pathogen	Inhibitory activity (AU/ml)			Mean ± SD
		80 °C	100 °C	121 °C	
BAL 0 A	*E. coli*	5,462.03±603.42	5,768.44±486.89	6,179.83±244.04	
	S. aureus	4,918.76 ±806.66	4,448.18 ±445.06	3,570.70± 375.57	
	Mean	5,190.39±703.18	5,108.31±834.86	4,875.27±1,456.88	5,057.99±996.77[a]
BAL 1 A	*E. coli*	5,130.97±231.42	6,046.17±47.57	5,670.63±166.19	
	S. aureus	4,200.01 ± 297.67	4,607.06 ± 546.19	3,777.21 ± 112.12	
	Mean	4,665.49±562.91	5,326.62±861.13	4,723.92±1,044.79	4,905.34±852.59[ab]
BAL 4 C	*E. coli*	3,627.433± 5.53	5,224.75± 291.09	5,064.29 ±30.45	
	S. aureus	4,360.67 ± 15.50	4,825.81 ± 534.99	5,495.48 ± 291.94	
	Mean	3,994.05±401.75	5,025.28±442.86	5,279.89±300.39	4,766.41±677.46[b]
Mean ± SD		4,616.65 ± 734.80[y]	5,153.40 ± 705.61[x]	4959.69 ± 1014.98[x]	

[a,b] Different superscripts in the same column show very significant differences (P<0.01)
[x,y] Different superscripts on the same line show very significant differences (P<0.01).

high temperatures. Bacteriocin derived from *Pediococcus pentosaceus* isolated from the Kalimantan rusip has strong heat stability even after being sterilized at 121°C for 15 minutes (Kusmarwati *et al.,* 2014). Similar results have been reported by Ogunbanwo *et al.* (2003) that the bacteriocin produced by *L. plantarum* F1 and *L. brevis* OG1 still showed activity at heating temperatures of 100°C for 10-30 minutes and heating at 121°C for 10-60 minutes. Another study stated that bacteriocin from *L.plantarum* ST16Pa was stable after being heated at 25-100°C for 2 hours and there was no reduction in activity after being treated at 121°C for 20 minutes at pH 6 (Todorov and Dicks, 2006; Todorov *et al.,* 2011). The results of research from Sankar *et al.* (2012) explained that the bacteriocin from *L.plantarum* isolated from cow's milk has stable stability at high temperature reaching 121°C. De Vuyst and Vandame (1994) stated that heat stability is caused by the presence of highly hydrophobic regions, stable cross-linking, and high glycine content. The persistence of antibacterial activity by bacteriocin, when given heat treatment, is suspected because bacteriocin is a short peptide that is stable to heat. In addition, due to the presence of certain amino acids in the bacteriocin which is able to maintain the bacteriocin structure from the influence of heat (Ray, 2004).

Stability of bacteriocin during storage

Storage is an important process in every manufacturing of a product. A good storage system will keep the product in its original condition and the quality is maintained. Conditions and storage time can affect the inhibitory activity of crude bacteriocin extracts from lactic acid bacteria (BAL 0 A, BAL 1 A, and BAL 4 C), so it is important to test the crude bacteriocin extract to determine the shelf life and storage conditions right. Stability of bacteriocin activity towards storage is presented in Table 4 and 5.

On day 0, bacteriocin activity at chiller storage and room temperature did not show much different activity, while storage in the freezer had a significantly lower activity (P<0.01) than bacteriocin stored at chiller and room temperature. Based on the type of lactic acid bacteria, bactericidal activity between BAL 0 A and BAL 4 C was almost the same, whereas for BAL 1 A had the highest highly significant activity (P<0.01) in the total storage.

On the 14th day, bacteriocin activity in the freezer, chiller, and room temperature storage showed significantly different activities (P<0.01). The total bacteriocin activity of three types of lactic acid bacteria showed optimum activity in chiller temperature storage, while the lowest activity of BAL 1 A has the highest highly significant total activity (P<0.01) against *E.coli* or *S.aureus*, whereas BAL 0 A is not too different from BAL 1 A and BAL 4 C, but BAL 4 C activity is lower than BAL 0 A and BAL 1 A.

The effect of storage conditions on each type of lactic acid bacteria has different effects. The greatest decrease of bacteriocin activity in BAL 0 A against *E.coli* or *S. aureus* is at the storage temperature of the chiller, while the smallest decrease in activity at the storage temperature is a freezer. The greatest decrease of bacteriocin activity in BAL 1 A was shown at room temperature storage, while the smallest decrease in activity was shown in chiller temperature storage. The greatest decrease of bacteriocin

Table 4. Bacteriocin stability of selected lactic acid bacteria during storage (day 0)

LAB Strain	Pathogen	Inhibitory activity (AU/ml)			Mean ± SD
		-8°C (freezer)	11°C (chiller)	29°C (room)	
BAL 0 A	*E. coli*	4,012.29 ± 563.76	5,242.02 ± 175.57	3,852.41 ± 502.94	
	S. aureus	4,260.82 ± 447.76	5,378.04 ± 147.20	4,959.63 ± 590.92	
	Mean	4,136.56±475.24	5,310.03±147.20	4,959.63±590.92	4,617.54±721.07[b]
BAL 1 A	*E. coli*	3,572.97 ± 59.46	5,087.43 ± 85.97	4,835.55 ± 23.26	
	S. aureus	5,856.83 ± 209.26	4,501.03 ± 149.66	5,881.33 ± 128.04	
	Mean	4,714.90±1,258.47	4,794.23±339.22	5,358.44±578.68	4,955.86±827.68[a]
BAL 4 C	*E. coli*	3,293.93 ± 162.11	4,923.63 ± 153.68	5,040.54 ± 176.72	
	S. aureus	3,814.63 ± 388.84	4,207.39 ± 63.49	5,303.25 ± 559.30	
	Mean	3,554.28±390.29	4,565.51±406.15	5,171.89±397.90	4,430.56±781.85[b]
Mean ± SD		4,135.25 ± 902.64[y]	4,889.92 ± 439.13[x]	4,978.78 ± 709.85[x]	

[a,b] Different superscripts in the same column show very significant differences (P<0.01)
[x,y] Different superscripts on the same line show very significant differences (P<0.01)

Tabel 5. Bacteriocin stability of selected lactic acid bacteria during storage (day 14)

LAB Strain	pathogen	Inhibitory activity (AU/ml)			Mean ± SD
		-8 °C (freezer)	11 °C(chiller)	29 °C (room)	
BAL 0 A	*E. coli*	3,890.63±116.44	4,578.38±405.43	3,205.99±290.05	
	S. aureus	3,792.86±531.84	4,509.56±487.64	5,332.45±322.40	
	Mean	3,841.74±348.47	4,543.97±402.85	4,269.22±1,196.57	4,218.31±770.03[ab]
BAL 1 A	*E. coli*	3,946.72±557.75	4,510.40±438.48	3,485.24±502.65	
	S. aureus	4,461.42±268.48	4,392.13±357.19	5,547.66±194.67	
	Mean	4,204.07±482.43	4,451.26±363.50	4,516.45±1,179.95	4,390.59±732.11[a]
BAL 4 C	*E. coli*	3,768.94±318.32	3,981.05±296.38	3,330.44±614.03	
	S. aureus	3,760.67±474.86	5,243.33±400.67	4,397.57±47.73	
	Mean	3,764.81±361.60	4,612.20±759.84	3,864.01±702.39	4,080.33±710.54[b]
Mean ± SD		3,936.87±425.99[z]	4,535.81±510.89[x]	4,216.56±1,025.82[y]	

[a,b] Different superscripts in the same column show very significant differences (P<0.01).
[x,y,z] Different superscripts on the same line show very significant differences (P<0.01).

activity in BAL 4 C against both types of pathogens was shown at room temperature storage as well, while the smallest decrease in activity was shown in freezer temperature storage, even the results tended not to experience decrease activity from the original conditions.

Based on the overall total bacteriocin activity of the three types of lactic acid bacteria both against *E.coli* or *S.aureus*, the results showed that the greatest decrease in bacteriocin activity was shown at room temperature storage which ranged from 762.22 AU/ml. Storage at freezer temperature showed the lowest decrease in bacteriocin activity, compared to the storage of chiller temperatures and room temperature which was 198.38 AU/ml. Whereas storage at chiller temperatures showed a decrease in bacteriocin activity lower than room temperature storage, but greater when compared with freezer temperature storage (354.11 AU/ml). So the optimum conditions for storage of crude bacteriocin extract for 14 days are best stored at low temperatures, in this study that is at freezer temperature.

Bacteriocin is a type of peptide that has a bactericidal function. This peptide is also one of the nutrients that are highly needed by both microorganisms or organisms. This crude bacteriocin extract if left or stored at room temperature, many microorganisms can grow to utilize this peptide for its nutrient source. Bacteriocin extract will be easily damaged and its activity can decrease, compared to being stored at low temperature, the possibility of microbes growing at low temperatures can last longer and decrease its activity also lower than at room temperature storage. Parker (2003) stated that temperature can affect the rate of change in the quality of a product related to the occurrence of chemical or biochemical reactions in the product. The higher the ambient temperature that affects the system, the chemical or biochemical reactions generally increases two times higher with each 10°C rise in temperature.

Research by Usmiati and Rahayu (2011) stated that at 12 weeks storage, storage at 4°C can minimize the percentage decrease in the inhibitory activity of bacteriocins extract powder from *Lactobacillus* sp. strain SCG 1223 against *E.coli*, compared to storage temperatures of 25-31°C. In contrast to the inhibition against *P.acidilactici*, the percentage of inhibitory activity decrease at storage at 4°C and 25-31°C at 12 weeks is relatively similar. In contrast to the research of Kusmarwati *et al.* (2014) that the bacteriocin produced by RK4 lactic acid bacteria was more stable after storage for 4 weeks at 37°C and 4°C, but was not detected after storage at -20°C. Research by Ogunbanwo *et al.* (2003) explained that the bacteriocin produced by *L.brevis* and *L. plantarum* still had stable activity after being stored for 60 days at -20°C, but the inhibitory activity decreased after being stored for 80 to 120 days at 37°C, which indicates that storage at cold temperatures is more appropriate way for bacteriocin from *L.brevis* and *L. plantarum*. Ogunbanwo and Okanlawon (2006) further explained that the immobilization of bacteriocin produced by *L. brevis* OG1 in calcium alginate gel and applied to the surface of cutaneous tissue in broiler meat can effectively reduce pathogenic bacteria by 21 days refrigerator storage (temperature 4°C). The effect of storage time and storage temperature on bacteriocin produced by *S. thermophillus, L. acidophilus, L. lactis,* and *P. acidilactici* showed a decrease in activity in line with increasing storage time and increasing storage temperature. Storage at -20°C and 4°C can maintain bacteriocin inhibitory activity for up to 6 weeks, while storage at 37°C only retains bacteriocin inhibitory activity for up to 4 weeks. The lowest inhibitory reduction was found in the storage treatment at -20°C, while the greatest decrease in activity was at a storage temperature of 37°C (Sumathi and Reetha, 2012). Seem likes with research by Obi (2015) that the bacteriocin produced by *Lactobacillus tucceti* CECT 5920 and *Lactobacillus mindensis* TMW decreased the inhibitory activity against *E.coli* 0157: H7 dan *S. aureus* NCTC 8325 during 14 days storage at room temperature.

Conclusions

Based on the results of the study it can be concluded that the bacteriocin activity of three types of lactic acid bacteria against *S.aureus* is greater than that of *E.coli*. The bacteriocin activity produced by BAL 0 A, BAL 1 A, and BAL 4 C tends to be stable to heating temperatures 80, 100, and 121°C, but decrease with increasing pH value (pH 3, 7, dan 10). The best bacteriocin activity is found at pH 3 (acid) and the heating

temperature of 100°C. The bacteriocin activity of three types of lactic acid bacteria was still good when stored at -8°C for 14 days.

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