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Research Article

Indigenous Bacteriocin of Lactic Acid Bacteria from “Dadih” a Fermented Buffalo Milk from West Sumatra, Indonesia as Chicken Meat Preservative

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Abstract

Background and Objective: The bacteriocin isolated from fermented buffalo milk from West Sumatra-Indonesia, called Dadih, can be considered as a natural and safe antimicrobial compound for food products. The objective of this research was to evaluate the antimicrobial activity of bacteriocin from Dadih and its effectiveness as a preservative in chicken meat. **Materials and Methods:** This study used experimental method followed by statistical analysis using 3 experiments with duplication including experiment of meat samples (0 and 10% bacteriocin), storage temperatures (7 and 26°C) and storage duration (0, 1, 2, 3, 4, 5, 6 days and 0, 6, 12 hrs). Each experiment consists of a bacteriocin test, antimicrobial activity assay, physicochemical measurement and storability. **Results:** From 10 LAB isolates successfully obtained from Dadih, two isolates with D₇ code and D₁₀ code had the highest antimicrobial activity, reaching 11.75 mm and 12 mm, respectively. The meat treated by 10% of bacteriocin gave the lower total microbial (3rd and 5th day) and total *E. coli* (5th day) at 7 and 26°C. The pH and water activity (a_w) values of chicken meat with 10% of bacteriocin showed lower values at 7 and 26°C. The application of bacteriocin to chicken meat was able to inhibit the microbial growth that was still below standard for 3 days at 7°C and 6 hrs at 26°C. **Conclusion:** Based on research, lactic acid bacteria isolated from buffalo milk curd produced bacteriocin compound which has antimicrobial properties. This bacteriocin showed potential as a natural preservative for chicken meat by inhibiting the growth of pathogen microorganisms.

Key words: Bacteriocin, dadih, chicken meat, lactic acid bacteria, antimicrobial activity, preservative

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTIONS

Poultry meat, particularly chicken meat, plays an important role in a human's diet and is considered as one of the best protein sources globally consumed. Due to the favorable conditions such as high a_w , appropriate pH and a rich source of nutrients for various types of microbes to grow in these foodstuffs, meat and other poultry products are very susceptible to damage¹. In addition, high water content and high water in the protein of chicken meat also lead to spoilage and accelerate the pathogenic bacteria to grow. Consequently, this food material only has a short shelf life.

The food industry generally uses various approaches to extend the shelf life of food products, such as the use of food additives or preservatives, which are hazardous at certain doses. Also, preservation using heat in high temperatures and the dry process can reduce the quality and also change the texture of food products². Refrigeration is also often used for preserving meat products because it can maintain the microbiological standard for meat quality. It, however, cannot guarantee that the use of refrigerator can positively impact on long shelf life for this meat products³.

The increase in demand for more natural and microbiological products, because of their safety for consumption, encourages considerable studies to find and develop new methods in food preservation. One of the proper techniques is the use of bacteriocin from Lactic Acid Bacteria (LAB) to control the growth of microorganisms in food products⁴. Bacteriocin is an antimicrobial peptide (AMPs) synthesized by certain bacteria through the ribosomal pathway^{5,6} and excreted extracellularly⁷. Antimicrobial compounds can extend the shelf life of chickens by inhibiting bacterial growth⁸.

Bacteriocin is a natural preservative potentially used for inhibiting the growth of pathogenic bacteria⁹. Among all microorganisms, the promising bacteria that can produce bacteriocin for bio preservatives is LAB¹⁰. This bacteria type is commonly found in fermented and non-fermented food products¹¹ and is safe for consumption. Furthermore, LAB also can produce a variety of antimicrobial compounds such as organic acids and bacteriocin¹². Bacteriocin from LAB has been successfully applied as a natural alternative for preserving food and as an antibiotic, because LAB was identified as GRAS (Generally Recognizes As Safe), which is given by the FDA (the American Food and Drug Agency)⁵, so it is safe for consumption.

Bacteriocin-producing LAB can be found in several fermented foods¹³. One of the Indonesian traditional foods that use the lactic acid fermentation process is Dadih. Dadih is fermented buffalo milk from West Sumatra and is usually

sold in traditional markets¹⁴. Dadih has a texture like soft cheese. Dadih is naturally fermented in a bamboo container at room temperature¹⁵ for 24-48 hrs.

Numerous LABs are found in dadih, therefore dadih is considered as a proper sample to isolate LAB. The research on the performance evaluation of bacteriocin as an antimicrobial for foodstuffs has been numerous reported. However, the study of bacteriocin isolated from LAB in Dadih is still overlooked. Thus, this study was aimed to evaluate the antimicrobial activity of bacteriocin from LAB in Dadih and its application for chicken meat preservation.

MATERIALS AND METHODS

Study area: This research was conducted in December 2019-March 2020 at Food Microbiology Laboratory, Faculty of Agro-Industrial Technology, Padjadjaran University, Bandung, Indonesia.

Microorganism: The indicator bacteria used were *E. coli* and *Salmonella* sp. obtained from the food microbiology laboratory, Faculty of Agro-Industrial Technology, Padjadjaran University. *Escherichia coli* and *Salmonella* sp. were maintained at Nutrient Agar (NA) and stored at 4°C.

Isolation of lactic acid bacteria: The sample, Dadih (from traditional markets in Bukittinggi, West Sumatra, Indonesia), was diluted with 0.85% NaCl solution serially from 10^{-1} to 10^{-8} , followed by transferred 1 mL of 10^{-6} to 10^{-8} dilution of sample suspension to a sterile petri dish. Subsequently, the MRS (deMan Rogosa and Sharpe) agar medium added 1% (w/v) CaCO_3 was poured to each dish and incubated at 37°C for 48 hrs. The isolates suspected as LAB was based on the formation of clear zones around the colony¹⁵. Colonies forming clear zones were then scratched 2-3 times on MRS agar to produce a single colony which was then identified qualitatively by Gram staining and catalase test.

Production of crude bacteriocin: Single-cell colony picked and inoculated into 5 mL of sterile MRS broth in a test tube and incubated at 37°C for 24 hrs. After that inoculating 2 mL of LAB inoculum into a test tube containing 18 mL of sterile MRS broth and incubated at 37°C for 9 hrs. According to modified Marie *et al.*¹⁶, bacteriocin extraction was initiated by centrifuged the broth culture in MRS medium at 7,000 rpm for 20 min at 4°C to separate the supernatant from the cell mass containing bacteriocin. After that supernatant was neutralized to pH 7 using NaOH 1N, then the supernatant was subsequently filtered using a membrane filter with a size of 0.22 μm to get a cell-free supernatant.

Antimicrobial activity of crude bacteriocin: The antimicrobial activity test of bacteriocin was carried out using the diffusion method. First of all, the indicator bacteria were prepared on the NA media, subsequently incubated at 37°C for 24 hrs. One of the colonies was added to a test tube containing 5 mL of NaCl 0,85% and compared with McFarland No. 3 with optical density (OD) of 0.755. Muller Hinton Agar (MHA) media in a petri dish was inoculated with 1 mL of indicator bacterial suspension. After the inoculum solution was diffused, a hole with a diameter of 6 mm was prepared in the media. The crude bacteriocin taken as much as 50 µL and put in diffusion well and left for 3 min at room temperature, then incubated at 37°C for 24 hrs. To indicate the activity of the crude bacteriocin, it could be seen from the appearance of clear zones around the diffusion well. Then the inhibitory unit activity is calculated by the formula according to Wong *et al.*¹⁷:

$$AU mL^{-1} = \frac{\text{Inhibitory zone area (mm}^2) - \text{Well area (mm}^2)}{\text{Volume of sample (mL)}}$$

Effect of adding crude bacteriocins to chicken meat quality:

Bacteriocin with a concentration of 10% (v/b) was sprayed on all parts of the 25 g chicken meat and waited for 30 min for bacteriocin to seep. After that, the chicken meat was packaged using sterile polypropylene (PP) plastic and was stored at refrigerator (7°C) and observed every 24 hrs for 6 day. On the other hand, the chicken meat stored at room temperature (26°C) was observed at 0, 6 and 12 hrs. The phytochemical (pH and a_w) and microbiological (Total Plate Count and *E. coli* detection) analysis were conducted to investigate the quality of meat.

Statistical analysis: All of the experiments in this research were carried out with two replicates and the results expressed as Mean ± standard deviation (SD). The regression was performed to observe the increase in the total number of microbes and *E. coli* during the storage of chicken meat. One-way ANOVA with Duncan's test was carried out on observations of the microbiological quality of chicken meat.

RESULTS

Isolation and characterization of lactic acid bacteria: The results of Lactic Acid Bacteria (LAB) isolation grown in the MRSA with 1% (w/v) CaCO₃ medium from 2 samples of dadih showed that 10 bacterial isolates were detected indicated with

the presence of a clear zone around the bacterial colony. The 10 bacterial isolates obtained were then characterized by the catalase test and bacterial Gram staining (Table 1).

The bacterial isolate shows a negative catalase test and Gram-positive bacteria could be assumed as a LAB isolate. According to the result, there were 7 isolates considered as LAB, viz. D₁, D₂, D₅, D₆, D₇, D₉ and D₁₀. Furthermore, the bacteriocin extraction was performed for these isolates.

Antimicrobial activity of crude bacteriocin: This study evaluated the bacteriocin antimicrobial activity against *E. coli* and *Salmonella* sp. After the test using the diffusion method was conducted, each isolate showed different inhibition of the test bacteria (Fig. 1).

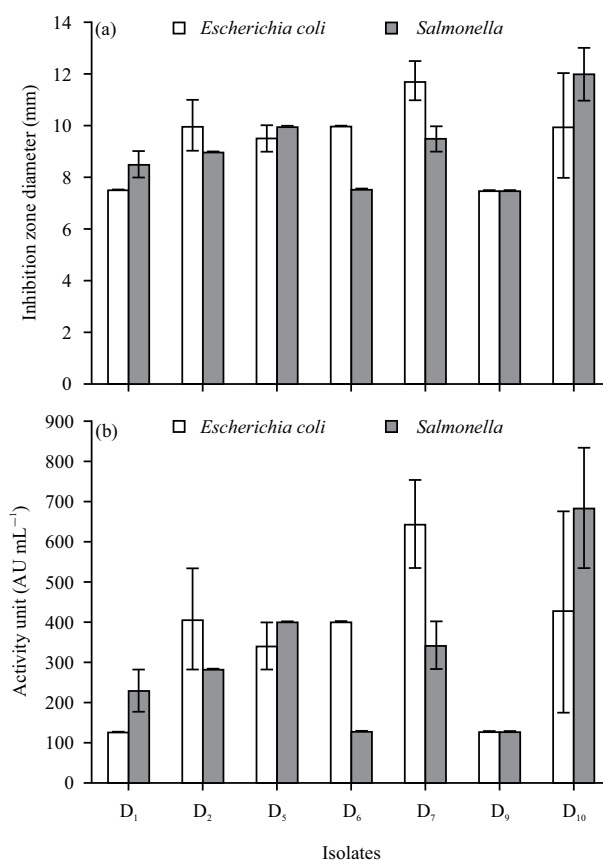


Fig. 1(a-b): Antimicrobial activity of crude bacteriocin to *E. coli* and *Salmonella* sp. (a) inhibition zone of crude bacteriocin against *E. coli* and *Salmonella* sp, (b) activity unit of bacteriocin against *E. coli* and *Salmonella* sp.

Table 1: Catalase and gram-test of lactic acid bacteria

Isolates	D ₁	D ₂	D ₃	D ₄	D ₅	D ₆	D ₇	D ₈	D ₉	D ₁₀
Catalase	-	-	-	-	-	-	-	-	-	-
Gram	+	+	-	-	+	+	+	-	+	+

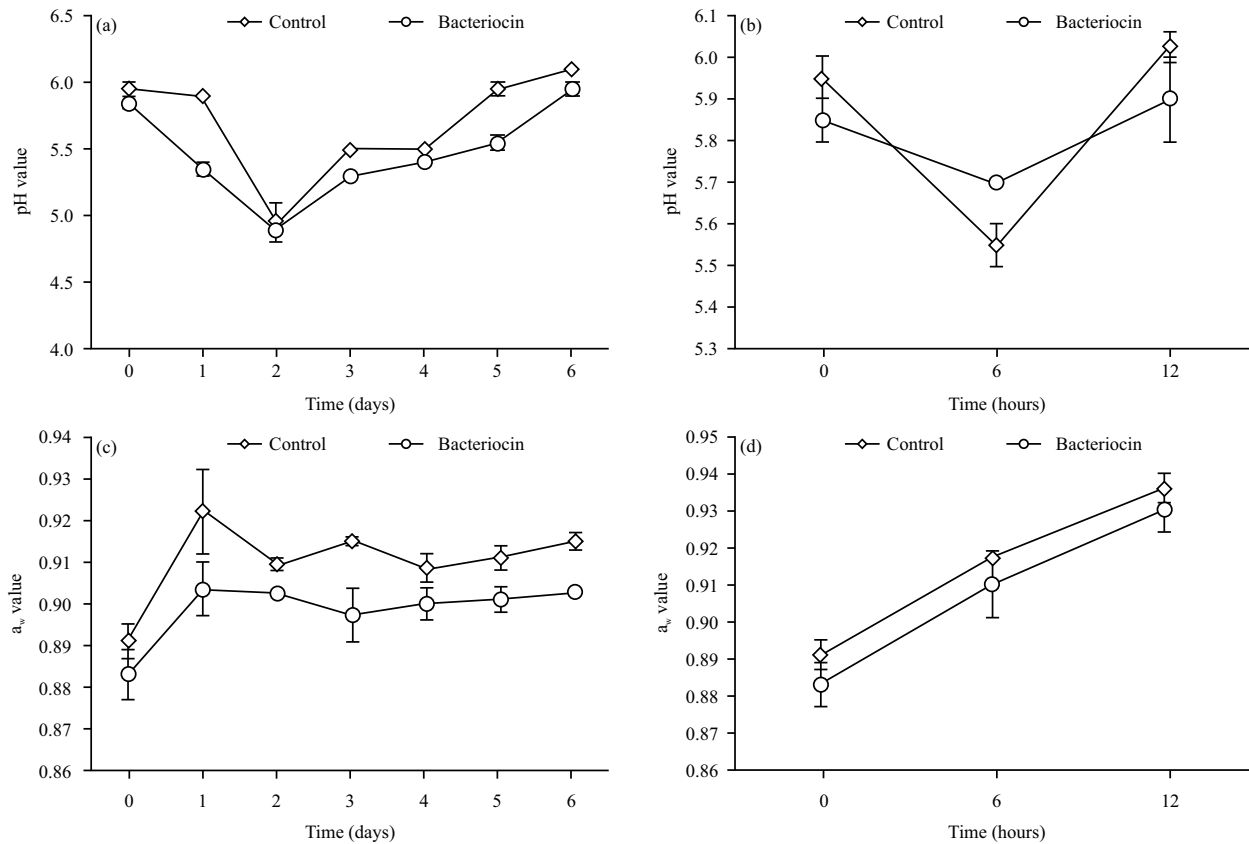


Fig. 2(a-d): (a) pH value of chicken meat during storage at 7°C, (b) 26°C, (c) Affinity water value of chicken meat during storage at 7°C and (d) 26°C

The results of the bacteriocin antimicrobial activity from 7 isolates showed different results. This might be influenced by the type of bacterial cell wall or the specific characteristics of the test bacteria. The bacteriocin from D₁ and D₁₀ isolates was more effective to inhibit *Salmonella* sp. than *E. coli*. While isolates D₂, D₅, D₆ and D₇ were more effective in inhibiting *E. coli*. D₉ isolates showed the same diameter of the inhibition zone in *Salmonella* sp. and *E. coli*. Inhibition zone diameters for each isolate showed different results. The size of the inhibition zone was influenced by several factors including the level of sensitivity of the test bacteria, the speed of diffusion of antimicrobial compounds and the concentration of antimicrobial compounds. The isolate with the D₁₀ code had a higher activity unit value than other isolates. Therefore, D₁₀ isolates were selected to be further tested to investigate their effect on the quality of chicken meat.

Effect of adding crude bacteriocins to chicken meat quality

Physicochemical parameters: The pH and a_w values of chicken meat during storage could be seen in Fig. 2.

According to Fig. 2a, the decrease in pH occurred started at the beginning of storage until the second day. There was an increase in pH from the second day to the sixth day of storage. The pH of control was higher than that with the treatment of bacteriocin addition from the beginning of storage to the sixth day of storage. While at room temperature (Fig. 2b), it showed a decrease in pH at 6 hrs of storage, then the pH value increased until 12 hrs of storage. The initial and final pH value of chicken meat with the addition of bacteriocin was lower than the control chicken meat but when the pH decreased at 6 hrs, the pH value of the control chicken meat was lower than the chicken meat with the addition of bacteriocin treatment.

Figure 2c and d showed the a_w value of chicken meat at two different temperatures. According to Fig. 2, chicken meat with the addition of bacteriocin treatment gave a lower a_w value than that of the control in both cold and room temperature storage. In cold temperature storage, the a_w value of chicken meat with the addition of bacteriocin treatment showed a more stable value than that of the control of chicken meat.

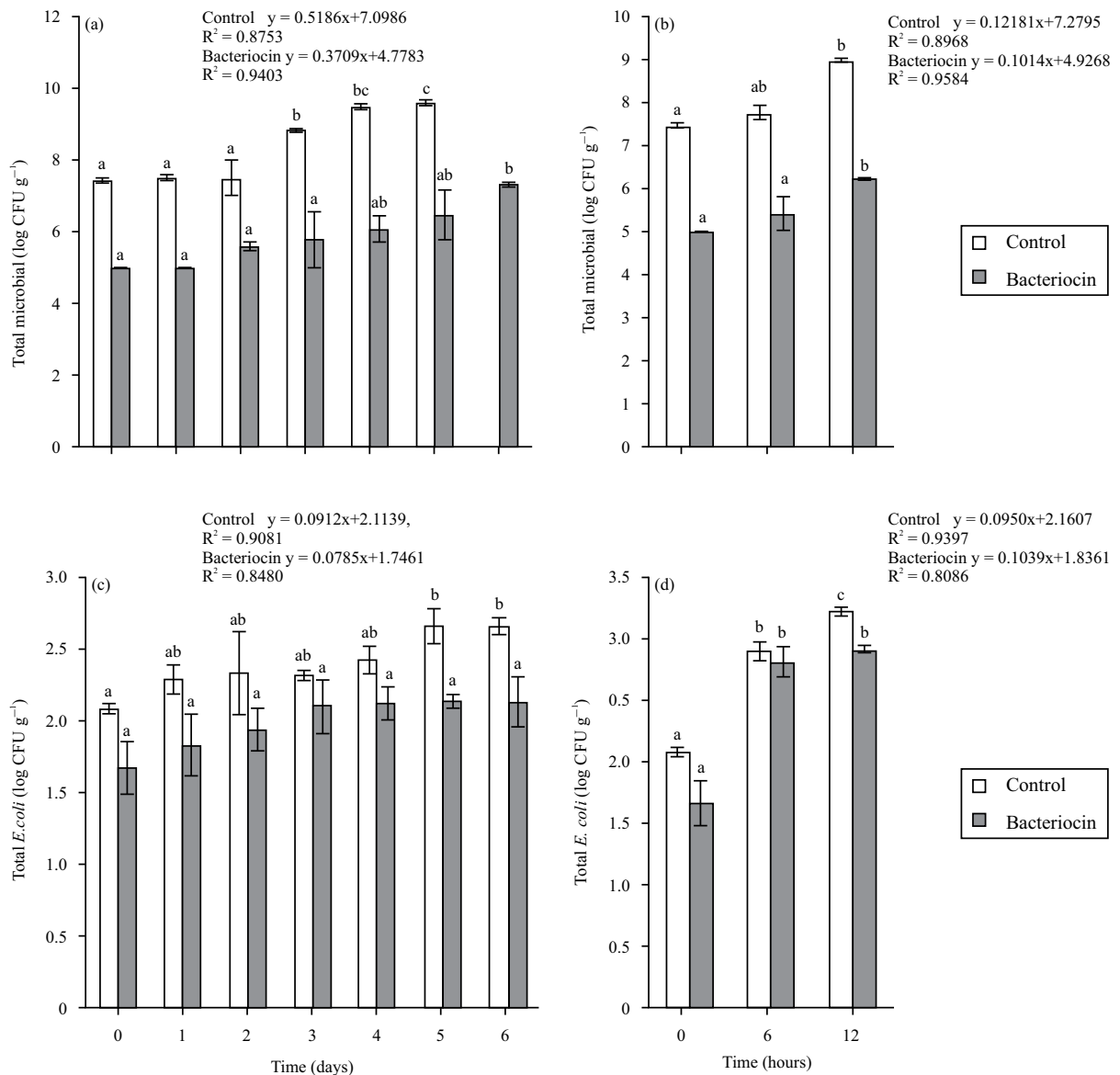


Fig. 3(a-d): (a) Total plate count of chicken meat during storage at 7°C, (b) 26°C, (c) Total *E. coli* of chicken meat during storage at 7°C and (d) 26°C

*6th day control shown too numerous to count/TNTC colonies

Microbiological parameters: Total microbes and *E. coli* in chicken meat were shown in Fig. 3. According to the results of the TPC showed that the total microbial number of chicken meat treated with bacteriocin was lower than that of control. In chicken meat at the beginning of the addition of bacteriocin showed a total microbial value of $5.00 \pm 0.000 \log \text{CFU g}^{-1}$ and an increase in total microbial approximately up to $2.31 \log \text{CFU g}^{-1}$ for 6 days stored at cold temperatures. Moreover, an increase in total microbes was by $1.22 \log \text{CFU g}^{-1}$ during 12 hrs of storage at room temperature.

While a total microbe of the control chicken meat before storage showed was $7.43 \pm 0.06 \log \text{CFU g}^{-1}$. The total microbe number for the sample stored at the cool temperature on the 6th day was too numerous to be counted but the total microbes for the sample stored at room temperature rose to $8.97 \pm 0.025 \log \text{CFU g}^{-1}$ after 12th hrs of storage.

Based on observations of TPC in cold storage (Fig. 3a), the total microbes in chicken meat treated with bacteriocin showed a significant increase starting on day 4, while in control chicken meat showed a significant increase starting on

day 3. At room temperature storage (Fig. 3b), total microbes in chicken meat treated with bacteriocin showed a significant increase at 12 hrs, while in control chicken meat showed a significant increase starting at 6 hrs of storage.

Total *E. coli* in cold storage (Fig. 3c) did not show a significant increase in chicken meat that was added by bacteriocin while in control chicken meat showed a significant increase starting on the 2nd day of storage. At room temperature storage (Fig. 3d), chicken meat with the addition of bacteriocin treatment showed a significant increase at 6 hrs then did not show a significant increase at 12 hrs. While the control chicken meat showed a significant increase at the 6th h and then again showed a significant increase at the 12th h of storage.

The results showed that the total value of *E. coli* in control chicken meat was higher than that of the sample treated by bacteriocin. The *E. coli* colony for control chicken meat before storage was $2.08 \pm 0.04 \log \text{CFU g}^{-1}$. The increase in total *E. coli* occurred for the sample of cold storage on the 6th day and room temperature storage at 12th h with the colony number up to 2.65 ± 0.060 and $3.22 \pm 0.035 \log \text{CFU g}^{-1}$, respectively. Meanwhile, chicken meat added by bacteriocin before storage showed a lower total value of *E. coli* at $1.66 \pm 0.180 \log \text{CFU g}^{-1}$, then increased to $2.13 \pm 0.175 \log \text{CFU g}^{-1}$ on the 6th day of storage at cold temperatures. The colony number continued to increase to $2.91 \pm 0.025 \log \text{CFU g}^{-1}$ at the 12th h of room temperature storage.

The results of the regression (Fig. 3a) showed that the slope value of control chicken meat (0.5186) was higher than that of chicken treated with bacteriocin (0.3709). This indicated that the average increase in the number of microorganisms in control chicken meat was higher than chicken meat with the addition of bacteriocin. Bacteriocin could also inhibit microbial growth in chicken meat stored at room temperature (Fig. 3b). Where based on the regression model, the slope value of chicken meat with bacteriocin treatment (0.12181) was lower than of the control chicken meat (0.1014).

Bacteriocin could inhibit the growth of *E. coli* in chicken meat stored at cold temperatures (Fig. 3c), where slope value in chicken meat with bacteriocin treatment was lower than in control chicken meat. Besides, at room temperature storage (Fig. 3d) showed that the increase in the average number of *E. coli* in chicken meat treated with bacteriocin was higher than the average increase in control chicken meat.

DISCUSSION

"Dadih" is fermented buffalo milk where the fermentation process involves lactic acid bacteria. Crude bacteriocin (which one of antimicrobial protein) could be extracted from lactic acid bacteria. According to this research, a crude bacteriocin from "Dadih" has antimicrobial activity, so it could be tested to inhibit the growth of microorganisms in chicken meat.

The selective medium used for isolation of lactic acid bacteria in this research were MRSa with 1% (w/v) CaCO_3 . The alkaline properties of CaCO_3 could neutralize and localize the acids from lactic acid bacteria. The colony shape of the LAB was typically white circular with an elliptical shape. This LAB was classified as facultative anaerobic bacteria with clear zones formed around the colony¹⁵. Lactic Acid Bacteria (LAB) was a group of bacteria that was unable to produce toxins, was classified as gram-positive bacteria containing low G+C, non-motile and could ferment sugar into lactic acid. The LAB had no cytochrome and was facultative anaerobic which could still grow in the presence of oxygen. In addition, LAB was a negative catalase, although in some cases this bacteria could be pseudo-catalase¹⁸. Hence, the bacterial isolate showing a negative catalase test and gram-positive bacteria could be assumed as a LAB isolate.

According to the results, there were differences in inhibition zones against *E. coli* and *Salmonella* sp. Several bacteriocins produced by lactic acid bacteria had a limited spectrum of pathogens in which Gram-positive bacteria had higher inhibitory performance than Gram-negative bacteria¹⁹. The inhibition zone differences indicated the presence of several types of bacteriocin with the different characteristics produced by lactic acid bacteria. The different types of bacteriocins led to different effectiveness against test bacteria. Mixtures of bacteriocins with different modes of action could produce greater inhibition as compared to mixtures of bacteriocins with the same mode of action²⁰.

Bacteriocin used as preservatives in food can be in the form of pure bacteriocin, partial purification, or in the form of cell-free supernatants²¹. In this study, it used crude bacteriocins from cell-free supernatants as chicken meat preservatives. According to the result, at the beginning of storage, the pH value of chicken meat initially decreased, both control and the samples with the addition of bacteriocin treatment in both cold and room temperature storage. The decline in pH value depicted the formation of organic acids, which originate from tissue glycolysis after the death of chickens²². The increase in pH observed during testing showed the level of meat damage because of protein breakdown and free amino acid production which led to the formation of alkaline compounds such as NH_3 and amines²³.

The result of a_w value showed that chicken meat with the addition of bacteriocin treatment had a lower a_w value than that of control. Water is a dipolar molecule, so water can be attracted to charged species such as proteins. Some of the water in muscle cells is bound to protein²⁴. In meat products, there is the ability to bind water by protein. Proteins that are damaged by proteolytic bacteria caused the decrease in the ability to bind water so that in meat an increase in free water causes a_w to increase. The addition of preservatives tends to reduce water activity. Based on the results of observations on a_w parameters showed that chicken meat with the addition of bacteriocin treatment has a lower a_w value compared to control chicken meat. In general, bacteria would be more difficult to grow on materials with lower a_w . So that the shelf life of the product added by bacteriocin would increase due to the antimicrobial activity of bacteriocin in addition to inhibiting the growth of spoilage and pathogenic bacteria could also inhibit the growth of proteolytic bacteria, therefore, a_w could be maintained at lower values. According to Arief *et al.*²⁵, the addition of bacteriocin to meatballs stored in cold temperatures could reduce the value of a_w until the third day of storage.

Based on observations of the quality of microorganisms, showed an increase in the total number of microbes both in TPC and in the total number of *E. coli* in the control sample showed a more significant increase than chicken meat with the addition of bacteriocin treatment both at storage at room temperature and at cold storage.

According to Pateiro *et al.*²⁶ and Badan Standardisasi Nasional²⁷, the total microbial limit recommended was around $6 \log \text{CFU g}^{-1}$ or 1×10^6 . The result showed that chicken meat treated with bacteriocin gave the lower total microbial number than that of controls. This could be caused by bacteriocin that became bactericidal towards some microbes so that the total number of microbes is lower. Besides, the addition of bacteriocin showed a slower increase in total microbes and could maintain the quality of chicken meat until the 3rd day in the refrigerator storage. In the same way, the storage at room temperature showed that the number of microbes in chickens which were treated with bacteriocin addition was lower than the control and still within the standard limit until the 6th h of storage.

The maximum limit of *E. coli* contamination according to Badan Standardisasi Nasional²⁷ was $1 \times 10^1 \text{CFU g}^{-1}$ ($1 \log \text{CFU g}^{-1}$). The results of this study for the total *E. coli* test on chicken meat with the addition of bacteriocin also showed lower numbers as compared to controls both for the storage

at the refrigerator and room temperature. However, the total amount of *E. coli* exceeded the standard limit in both treatments. The bacteriocin added to the chicken was crude, so the concentration of bacteriocin in the cell-free supernatants was unable to be certainly determined. The bacteriostatic effect is directly proportional to the concentration of antimicrobial substances⁸. The higher the concentration of antimicrobial substances was added, the higher the bacteriostatic effect was.

The addition of bacteriocin was able to reduce the growth of pathogen bacteria on chicken meat. Our results suggest a possibility that bacteriocin from "Dadih" to be applied as chicken meat bio preservative and could be combined with other preservative methods. Further study can be continued by conducting molecular identification of the isolated lactic acid bacteria, as well as purifying the extracted bacteriocin. The application of bacteriocin to food can be combined with other antimicrobial or with other preservation techniques.

CONCLUSION

In this study, 10 isolates of lactic acid bacteria isolated from "dadih" gave the highest antimicrobial activity in which the inhibition zone diameter of *Escherichia coli* (D_7) and *Salmonella* sp. (D_{10}) was 11, 75 and 12 mm, respectively. Bacteriocin test of chicken meat showed that the pH and a_w values were lower both in cold and room temperature than the chicken meat added with bacteriocin. The treatment by adding bacteriocin had more stable a_w and pH. Moreover, the addition of bacteriocin showed a slower increase in total microbes and total *E. coli*. The application of bacteriocin to chicken meat could inhibit total microbial growth and be still below standard until the 3rd day in the refrigerator storage and the 6th h of storage at room temperature.

SIGNIFICANCE STATEMENT

This study discovers the possible antimicrobial effect of bacteriocin indigenous from lactic acid bacteria isolated from "Dadih" that can be beneficial for alternative preservatives in chicken meat and other food. This study will help the researcher to uncover the novel bacteriocin from lactic acid bacteria indigenous isolated from "Dadih" through characterizing physicochemical properties and abilities as antimicrobials. Thus, a new finding on this indigenous bacteriocin has potency as natural preservatives especially for application on chicken meat.

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