Antimicrobial activity of bacteriocin like inhibitory substance (BLIS) and lactic acid bacteria (LAB) isolated from traditional fermented buffalo milk from West Sumatra, Indonesia

F Hayati, T Yuliana and T Rialita
Department of Food Technology, Faculty of Agro-Industrial Technology, Padjadjaran University, Sumedang, West Java, Indonesia

Email: fitrahayati118@gmail.com

Abstract. Food preservation by chemical preservatives addition is not recommended in large quantities. However, lactic acid bacteria (LAB) metabolites can be used as a safe alternative. LAB can produce a type of antimicrobial peptide known as bacteriocin. One source of LAB that can be used as a bacteriocin-producing candidate is dadih, a traditional fermented buffalo milk product from West Sumatra, Indonesia. In this study, eight bacterial isolates isolated from dadih were identified using the catalase test and Gram straining and obtained 6 isolates that were taken as LAB. An antimicrobial activity test was carried out using the diffusion method on LAB liquid isolates suspension and crude bacteriocins from the six isolates. The isolates with the highest antimicrobial activity were DK8, the diameter of inhibiting zone of liquid isolates was 15.75 mm (Salmonella sp.), 14.25 mm (Escherichia coli), and 12.25 mm (Staphylococcus aureus). Meanwhile, the diameter of crude bacteriocin inhibition of DK 8 isolates was 8 mm (Salmonella sp.), 8 mm (E. coli), and 8.5 mm (S. aureus). Furthermore, 16S rRNA sequencing confirmed DK8 as Lactobacillus pentosus.

1. Introduction
Pathogenic bacteria cause damage to food and foodborne diseases. Therefore, it is necessary to prevent them from damaging food by increasing the quality and shelf life of the food. In addition, consumer demand for safe preservatives is growing due to the harmful effects of using the chemical compound as preservatives [1]. As consequence, the food industries use alternative preservatives which safer than chemical preservatives such as bacteriocin produced by lactic acid bacteria [2].

Strains of lactic acid bacteria can produce antimicrobial substances (organic acids and bacteriocins) and have the potential to be used as food preservatives [3]. During the fermentation process, most lactic acid bacteria produce various components such as organic acids, hydrogen peroxide, diacetyl, acetaldehyde, carbon dioxide, polysaccharides, and protein compounds called bacteriocins or bactericinogenic peptides. These metabolites have an antimicrobial activity to inhibit spoilage bacteria and pathogenic bacteria, thereby helping maintain the nutritional quality of food for longer shelf life. Due to the "Generally Recognised as Safe" (GRAS) status, the use of LAB or its metabolites as preservatives in food has increased in recent years [4].
Bacteriocin-producing LAB can be found in several fermented foods [5]. One of the traditional Indonesian foods that use the lactic acid fermentation process is dadih. Dadih, fermented buffalo milk from West Sumatra, is naturally fermented in a bamboo container at room temperature for 24-48 hours [6]. Numerous LABs are found in dadih, and is considered as a proper sample to isolate LAB. The research on the performance evaluation of bacteriocin as an antimicrobial for food has been numerous reported. However, the study of bacteriocin isolated from LAB in dadih is still overlooked. Thus, this study was aimed to obtain LAB isolates from dadih as a candidate for producing bacteriocin inhibitory substances which can be developed as an alternative food preservative [7].

2. Material and methods

2.1. Material

Dadih was purchased from traditional markets in Bukittinggi, West Sumatra, Indonesia. The indicator bacteria used were E. coli and Salmonella sp. and S. aureus obtained from the food microbiology laboratory, FTIP, Padjajaran University.

2.2. Methods

All of the experiments in this research were carried out with two replicates, and the results were expressed as mean ± standard deviation (SD).

2.2.1. Isolation and characterisation of lactic acid bacteria. Lactic acid bacteria (LAB) were isolated by serial dilution method [8]. Isolation is carried out using MRSA containing CaCO₃ (0.3% v/v) as preliminary isolation and screening medium for LABs from samples, incubated at 28°C for 48. The selection of isolates suspected of being LAB was based on forming a clear zone around the colony. The colonies that formed the clear zone were then characterised by Gram staining and catalase test. Pure isolates that were Gram (+) and catalase (-) were LAB isolates [9].

2.2.2. Crude bacteriocin extraction. The procedure for bacteriocin extraction was, 10 % of LAB cells were inoculated into MRSB medium and propagated for 18 hours. Cells were harvested by centrifugation at 6000 rpm for 10 minutes at 4°C. The supernatant obtained was then filtered using 0.20µm millipore. The filtered supernatant was neutralised with NaOH to a pH of about 6.5 to eliminate the effects of organic acids [10]. Lactic acid bacteria culture suspension that has been propagated for 18 hours and crude bacteriocin will then be tested for their inhibitory activity against indicator bacteria to determine their antimicrobial activity.

2.2.3. Antimicrobial test. Antimicrobial activity was tested using three indicator bacteria: E. coli, Salmonella sp., and S. aureus. Antimicrobial testing was carried out by modifying Emmawati et al., (2015) procedure. The test was carried out by placing 50 µL indicator bacteria cultures which had been equated with Mc Farland no. 0.5, spread on a petri dish containing MHA media. A 6 mm well was made of agar, and 50 µL of the sample was inoculated into the well. After 24 hours incubation at 37°C, the diameter of the inhibition zone was measured from 3 different sides.

2.2.4. Identification of lactic acid bacteria. The isolates with the highest antimicrobial activity were then identified molecularly to see the structure of their DNA sequences using 16S rRNA gene amplification [12]. In this study, the primers used for sequencing were forward primer 785F 5’ (GGA TTA GAT ACC CTG GTA) 3’ and reverse primer 907R 5’ (CCG TCA ATT CMT TTR AGT TT) 3’. The sequencing results were analysed using the MEGA software. Then BLAST was performed by equating the base sequence with the NCBI genebank data.
3. Result and discussion

3.1. Isolation and characterization of lactic acid bacteria

The results of LAB isolation from 2 samples of dadih showed that eight bacterial isolates were detected, indicated with the presence of a clear zone around the bacterial colony. The eight bacterial isolates obtained were then characterised by a catalase test and bacterial Gram staining. The results of the characterisation of 8 isolates can be seen in Table 1.

| Isolate code | Catalase test | Gram staining | Shape of cell |
|--------------|---------------|---------------|---------------|
| DK1          | (-)           | (+)           | Basil         |
| DK2          | (-)           | (-)           | Basil         |
| DK3          | (-)           | (+)           | Basil         |
| DK4          | (-)           | (+)           | Basil         |
| DK5          | (-)           | (+)           | Basil         |
| DK6          | (-)           | (-)           | Basil         |
| DK7          | (-)           | (+)           | Basil         |
| DK8          | (-)           | (+)           | Basil         |

Testing of lactic acid bacteria, which involves morphological and physiological assessment, was done by Gram staining and catalase test. The catalase test is used to determine the presence of the catalase enzyme using 3% \( \text{H}_2\text{O}_2 \) [13]. Bacteria assumed to be lactic acid bacteria are bacteria that show Gram-positive and catalase-negative [3]. LAB is usually non-motile, non-sporulating [14]. The bacterial isolate showing a negative catalase test and gram-positive bacteria could be expected as a LAB isolate. According to the result, there were six isolates accepted as lactic acid isolates. Such DK 1, DK3, DK4, DK5, DK6, and DK8. The crude bacteriocin was then extracted from these six isolates.

3.2. Antimicrobial activity of lactic acid bacteria suspension and crude bacteriocin

Lactic acid bacteria suspension and crude bacteriocins were then tested for antimicrobial activity. To indicate the antimicrobial activity, it could be seen from the appearance of clear zones around the diffusion well. The results are shown in Table 2. This study evaluated the bacteriocin antimicrobial activity of three test bacteria, \( E. \text{coli} \), \( \text{Salmonella sp.} \) and \( \text{Streptococcus aureus} \). After the test using the well diffusion method, each isolate showed different inhibition of the test bacteria. The results of the bacteriocin antimicrobial activity from 6 isolates showed different results. This might be influenced by the type of bacterial cell wall or the specific characteristics of the test bacteria.

| Isolates code | Samples   | Inhibit zone (mm) |
|---------------|-----------|-------------------|
|               |           | \( \text{Salmonella sp.} \) | \( E. \text{coli} \) | \( S. \text{aureus} \) |
| DK1           | LAB isolates | 12.75 ± 0.75      | 12.75 ± 0.25      | 14.25 ± 0.25      |
|               | Crude Bacteriocin | 7.50 ± 0.50        | 7.00 ± 0.00        | 8.00 ± 0.00        |
| DK3           | LAB isolates | 14.00 ± 0.00      | 12.00 ± 2.00      | 14.00 ± 0.50      |
|               | Crude Bacteriocin | 7.00 ± 0.00        | 7.00 ± 0.00        | 8.00 ± 0.00        |
| DK4           | LAB isolates | 13.00 ± 0.50      | 11.50 ± 1.00      | 12.00 ± 0.50      |
|               | Crude Bacteriocin | 8.25 ± 0.25        | -              | 8.00 ± 0.00        |
| DK5           | LAB isolates | 13.25 ± 1.25      | 13.50 ± 1.50      | 9.50 ± 0.50       |
|               | Crude Bacteriocin | -              | -          | 8.50 ± 0.50        |
| DK6           | LAB isolates | 14.00 ± 1.00      | 16.25 ± 0.25      | 14.25 ± 1.75      |
|               | Crude Bacteriocin | 8.25 ± 0.25        | 8.00 ± 0.00        | 8.00 ± 0.00        |
During lactate fermentation, LAB produces various compounds such as organic acids, diacetyl, hydrogen peroxide, and bacteriocins. This product of LAB metabolism is known to have beneficial effects, such as extending shelf life and inhibiting the growth of pathogenic organisms [15]. Lactic acid bacteria have an important role in inhibiting the growth of pathogenic bacteria with a broad spectrum. Numerous studies on the antimicrobial activity of LAB liquid isolates have been carried out. Several liquid isolates of lactic acid bacteria isolated from fresh camel milk showed a significant inhibitory effect on pathogenic bacteria. Strains of \( \text{E. lactis} \) and \( \text{L. plantarum} \) showed a strong inhibitory effect against \( \text{S. aureus} \), \( \text{E. coli} \), and \( \text{B. cereus} \). Meanwhile, the \( \text{Lactococcus lactis} \) strain showed a normal inhibitory effect against \( \text{E. coli} \) [3]. Study on the antimicrobial testing of liquid isolate \( \text{Lactobacillus plantarum} \) from Mandai (fermented inner shell of \( \text{Cempedak} \)) against enteric bacteria, antimicrobial activity showed strong activity against \( \text{L. monocytogenes} \), \( \text{E. faecalis} \), \( \text{B. cereus} \), \( \text{E. coli} \) and \( \text{S. Typhimurium} \) bacteria [11]. In addition, 9 out of 12 LAB isolates isolated from “\( \text{dadih} \)” showed an inhibition zone against \( \text{Listeria monocytogenes} \) of more than 3.5 mm [16].

LAB is a Gram-positive bacterium and the antimicrobial activity in LAB is due to the presence of various metabolites that act as antimicrobials. The antimicrobial activity of LAB against pathogens is species and strain-dependent. The antimicrobial activity of LAB is mainly due to the production of one or more active metabolites during its growth, such as organic acids, hydrogen peroxide, and bacteriocins [17]. One of the LAB metabolites is bacteriocin which is an antimicrobial peptide. Antimicrobial peptides produced by Gram-positive bacteria usually have a broad antimicrobial spectrum that can inhibit the growth of both Gram-positive and Gram-negative bacteria [18].

The measurement of the inhibitory activity against Gram-negative bacteria (as defined by the diameter of the well) was classified as follows: weak inhibitory strength (0-3 mm), medium (3-6 mm), strong (6-9 mm), and very strong (>9 mm) [11]. The diameter of the well in the antimicrobial activity test was 6 mm so that the inhibitory strength could be classified as follows: weak (6-9 mm), medium (9-12 mm), strong (12-15 mm), and very strong (>15 mm).

The results showed that LAB liquid isolates solution and crude bacteriocins had inhibitory activity against Gram-positive (\( \text{S. aureus} \)) and Gram-negative (\( \text{E. coli} \) and \( \text{Salmonella sp.} \)) bacteria. The observations showed that the inhibitory activity of crude bacteriocins against \( \text{S. aureus} \) was higher than the inhibitory activity of \( \text{E. coli} \) and \( \text{Salmonella sp.} \). Crude bacteriocins of all isolates showed inhibitory activity against Gram-positive bacteria. In contrast, bacteriocins from several isolates did not show activity against \( \text{E. coli} \) and \( \text{Salmonella sp.} \), which is a group of Gram-positive bacteria. Species type, strain, and Gram reaction (positive with negative) can have a significant effect. For example, hydrophobic antimicrobial activity can be inhibited by Gram-negative bacteria, which can screen antimicrobials due to the presence of an outer membrane lipopolysaccharide layer [19]. Very few bacteriocins have been reported to inhibit Gram-negative bacteria due to their narrow spectrum of action. Such characteristics often limit the potential of bacteriocins in food preservation and safety, and therefore, research on bacteriocins against Gram-positive and Gram-negative bacteria is very useful [4].

Based on the observations, the antimicrobial activity of LAB liquid isolates had stronger activity against Gram-negative bacteria (\( \text{E. coli} \) and \( \text{Salmonella sp.} \)) than Gram-positive bacteria (\( \text{S. aureus} \)). This is because the main metabolite produced by lactic acid bacteria is lactic acid which has a pH range of 3.5-4.5. Meanwhile, the minimum pH for the growth of pathogenic bacteria varies. The bacteria tested were \( \text{E. coli}, \text{Salmonella sp.}, \) and \( \text{S. aureus} \). The optimum pH for the growth of these three bacteria is neutral. While the minimum pH varies, the minimum pH of \( \text{E. coli} \) is 4.4; \( \text{Salmonella sp.} \) has a minimum pH of 4.05-5.4; while the minimum pH of \( \text{S. aureus} \) is 4.0 [20]. \( \text{S. aureus} \) bacteria have the lowest minimum pH; thus, \( \text{S. aureus} \) bacteria are more resistant to acid than \( \text{E. coli} \) and \( \text{Salmonella sp.} \) bacteria.

The antimicrobial mechanism by low pH, i.e., undissociated lipophilic acid molecules, can cross the membrane freely by moving from a low pH external environment where the equilibrium favours undissociated molecules to a high pH cytoplasm (about 7.5 in neutrophils). At higher pH, the equilibrium
shifts in the direction of the dissociating molecules so that the ionised acid produces protons which will tend to acidify the cytoplasm and split the pH component of the proton-motive force. The cell will try to maintain its internal pH by neutralising or expelling the leaking protons, which will slow the growth because it diverts energy from growth-related functions. Suppose the external pH is low enough and the extracellular acid concentration is high. In that case, the load on the cell becomes too high, the cytoplasmic pH drops to a level where growth cannot occur, and the cell eventually dies [20].

Overall, the antimicrobial activity of the LAB isolates showed higher activity than crude bacteriocins. This could be due to the presence of bacterial colonies in the LAB broth isolate so it was still possible for LAB to produce metabolites during incubation; thus, the number of metabolites would be higher. LAB produces organic acids such as lactic acid, which can simultaneously lower pH [14]. In comparison, crude bacteriocin has a pH that is close to neutral due to the addition of Sodium Hydroxide to neutralise the acid metabolites contained in the supernatant; hence, the results showed that the crude bacteriocin had lower inhibitory activity.

DK8 isolate showed the most inhibition activity, both the activity of LAB isolates and crude bacteriocins. The DK8 isolate was further identified molecularly using 16s rRNA sequencing with primers 785F and 907R. The results demonstrated that the isolates were similar to Lactobacillus pentosus (code: NR_029133.1) with a similarity level of 99%.

4. Conclusion
There were eight bacterial isolates isolated from dadih. Following identification using catalase test and Gram staining, there were six isolates expected to be LAB. The antimicrobial activity test was carried out using the well diffusion method on LAB liquid isolates and crude bacteriocins from the six isolates. Isolates with the highest antimicrobial activity were DK8, the diameter of inhibiting zone of liquid isolates was 15.75 mm (Salmonella sp.), 14.25 mm (Escherichia coli), and 12.25 mm (Staphylococcus aureus). Meanwhile, the diameter of crude bacteriocin inhibition of DK8 isolate was 8 mm (Salmonella sp.), 8 mm (E. coli), and 8.5 mm (S. aureus). Furthermore, 16S rRNA sequencing confirmed DK8 as Lactobacillus pentosus.

References
[1] Kumariya R, Kumari A, Rajput S, Sood S K and Akhtar N 2019 Microbial pathogenesis bacteriocins: classification, synthesis, mechanism of action and resistance development in food spoilage causing bacteria Microb. Pathogens. 128 171–7
[2] Parma N, De Castilho A, Dimitrov S, Licursi L, Bersot S and Augusto L 2020 Inhibition of listeria monocytogenes in fresh sausage by bacteriocinogenic Lactobacillus curvatus UFV-NPAC1 and its semi-purified bacteriocin LWT - Food Sci. Technol 118 108757
[3] Sharma A, Lavania M, Singh R and Lal B 2020 Saudi Journal of biological sciences identification and probiotic potential of lactic acid bacteria from camel milk Saudi J. Biol. Sci. 28 3 1622-32
[4] Patel A, Shah N, Ambalam P, Prajapati J B, Holst O and Ljungh A 2013 Letter to the editor antimicrobial profile of lactic acid bacteria isolated from vegetables and indigenous fermented foods of india against clinical pathogens using microdilution method Biomed. Environ. Sci. 26 9 759–64
[5] Xinran L, Ma H, Sun M, Lin Y, Bai B, Li J Zhang B 2018 A novel bacteriocin DY4-2 produced by Lactobacillus plantarum from cutlass fish and its application as bio-preservative for the control of Pseudomonas fluorescens in fresh turbot (Scophthalmus maximus ) fillets Food Control 89 22–31
[6] Sunaryanto R and Marwoto B 2013 Isolasi, identifikasi, dan karakterisasi bakteri asam laktat dari dadih susu kerbau (Isolation, identification, and characterization of lactic acid bacteria from buffalo milk curd) J. Sains dan Teknol. Indones. 14 3 228–33 [In Indonesian]
[7] Yuliana T, Hayati F, Cahyana Y, Rialita T, Mardawati E, Harahap B M and Safitri R 2020 Indigenous Bacteriocin of lactic acid bacteria from ‘dadih’ a fermented buffalo milk from
West Sumatra, Indonesia as chicken meat preservative 23 12 1572-80

[8] Yi L, Dang J, Zhang L, Wu B, Liu B and Liu X 2016 Purification, characterisation and bactericidal mechanism of a broad spectrum bacteriocin with antimicrobial activity against multidrug-resistant strains produced by Lactobacillus coryniformis XN8 Food Control 67 53–62

[9] Ying Z, Yang J, Ying L, Wu Y, Yang Z, Wang Y, Sun L, Deng Q, Gooneratne R, Xiao L and Zhou Y 2020 A novel bacteriocin PE-ZYB1 produced by Pediococcus pentosaceus zy-B isolated from intestine of Mimachlamys nobilis: Purification, identification and its anti-listerial action LWT - Food Sci. Technol 118 108760

[10] Cizeikiene D, Juodeikiene G, Paskevicius A and Bartkiene E 2013 Antimicrobial activity of lactic acid bacteria against pathogenic and spoilage microorganism isolated from food and their control in wheat bread Food Control 31 2 539–45

[11] Emmawati A, Sri B, Suryaatmadja L, Nuraida L and Syah D 2015 Characterisation of lactic acid bacteria isolates from mandai function as probiotic Agritech 35 2 146–55

[12] Ahmad V, Khan M S, Jamal Q M S, Alzohairy M A, Al Karaawi M A and Siddiqui M U 2017 Antimicrobial potential of bacteriocins: in therapy, agriculture and food preservation Int. J. Antimicrob. Agents 49 1 1–11

[13] Matti A, Utami T, Hidayat C and Rahayu E S 2019 Isolation, screening, and identification of proteolytic lactic acid bacteria from indigenous chao product bacteria from indigenous chao product J. Aquat. Food Prod. Technol. 28 7 781-793

[14] Ringøa E, Schillinger U and Holzapfel W Antimicrobial activity of lactic acid bacteria isolated from aquatic bacteria in aquaculture Biology Grow. Anim. 2 2005 418-53

[15] Bryan C A O, Crandall P G, Ricke S C and Ndahetuye 2015 J B, 6 - Lactic acid bacteria as antimicrobials in food products: types and mechanisms of action (Elsevier Ltd)

[16] Pato U, Yusuf, Fitriani S and Jonnadi N I A N 2020 Inhibitory activity of crude bacteriocin produced by lactic acid bacteria isolated from dadih against Listeria monocytogenes 21 4 1295–302

[17] Rahmeh R, Akbar A, Kishk M, Shajan A and Akbar B 2019 Distribution and antimicrobial activity of lactic acid bacteria from raw camel milk New Microbes New Infect. 30 100560

[18] Galvez A, Maqueda M, Martinez-Bueno M and Valdivia E 1989 Bactericidal and bacteriolytic action of peptide antibiotic As-48 Against gram-positive and gram-negative bacteria and other organism Res. Microbiol. 140 57–68

[19] Davidson P M, Sofos J N and Branen A L 2005 Antimicrobials in Food (Boca Raton, Florida, US: CRC Press, Taylor & Francis Group)

[20] Adams M R and Moss M O 2008 Food Microbiology Third Edit. (Guildford, UK: RSC Publishing)