Sourcing new potential bacteriocin-producing bacteria from dangke, ethnic cheese of Enrekang, Indonesia

N J N Djide, R M Asri, and N Djide
Faculty of Pharmacy, Hasanuddin University, Jl. Perintis Kemerdekaan No.10, Makassar, Sulawesi Selatan, 90245, Indonesia

Email: nanajuniarti@unhas.ac.id

Abstract. Bacteriocins are bioactive peptides produced by microorganisms. Sourcing of new bacteriocin-producing bacteria is essential as bacteriocin production depends on the microbial strain. One of potential sources for bacteriocin-producing microorganisms is Dangke—a traditional cheese of Enrekang, South Sulawesi, Indonesia. Dangke contains compounds that can support the growth of lactic acid bacteria (LAB)—one of the well-known bacteriocin-producing bacteria. Specifically, this study focused on finding new strains of bacteriocin-producing LAB from dangke and screening its activity against pathogenic bacteria. LAB were isolated from cow and buffalo dangke using MRSA-CaCO3 1% media. The bacterial isolates obtained were identified based on 16S rRNA analysis. Fermentation was done in MRSB media in 37°C for 72 hours then concentrated with ammonium sulphate 80%, followed by dialysis. Crude bacteriocins were tested against Escherichia coli and Staphylococcus aureus. LAB strain B3B1 and S2A1 were successfully isolated from Dangke and were identified as Lactobacillus fermentum strain NBRC 15885 (identities 99%) and Weissella confusa strain JCM 1093 (identities 99%). Crude bacteriocin of L. fermentum strain NBRC 15885 was active against S. aureus with inhibition zone of 11.34 ± 2.97 cm. Based on the result, isolate B3B1 identified as Lactobacillus fermentum from buffalo dangke has potential in producing bacteriocin. Further studies of production, optimization, characterization and screening of other bioactivity of the bacteriocin are suggested.

1. Introduction
Bacteriocin is a group of ribosomally synthesized bioactive peptides produced by both Gram-positive and Gram-negative bacteria [1]. There are two types of bacteriocin: a classic type of bacteriocin that active against bacteria related to its producer (narrow-spectrum) and the second type which is active against wide range of microorganism (broad-spectrum) [1]. Bacteriocin have long been used in biopreservation in the food industry and shown emerging potential as antiviral, anticancer, promoting plant growth [1], skin care [2] and an alternative to antibiotics [3].

Bacteriocins are diverse in size, targets, mechanism of action and immunity [4] and their production depends on culture conditions as well as the producer strain [5]. Bacteriocin produced by Gram-positive bacteria are more varied than those produced by Gram-negative bacteria [4].

Lactic acid bacteria have been known as bacteriocin-producing bacteria. The characteristics produced by LAB are categorized as Generally Recognized as Safe Substance (GRAS), usually tolerant against pH and heat and has a broad spectrum against microorganism [6]. LAB also is playing...
candidates for probiotics — living microorganisms that can provide health benefits to their causes when administered and adequate amounts [7].

A potential source of lactic acid bacteria is dangke—an ethnic cheese made of buffalo milk or cow milk—from Enrekang, South Sulawesi, Indonesia [8]. Dangke is made by heating milk with low heat to boil then papaya sap is added to agglomerate the milk. The formed clump is inserted into a mold made of coconut shell sauce then pressed to separate the liquid [9]. Dangke is selected as a source for isolation because it contains compounds that can support the growth of LAB and has long been consumed by the community so it is relatively safe. Nur et al. [8] managed to isolate two strains of potentially probiotic lactic acid bacteria from dangke, Lactobacillus plantarum and L. fermentum. Since microbial strain influences bacteriocin produced by microorganisms, we notice its potential as the source for bacteriocin-producing bacteria. This study focused on finding new strains of bacteriocin-producing LAB from dangke and screening its activity against pathogenic bacteria.

2. Materials and Methods

2.1. Isolation and Identification of the Isolates.

2.1.1. Isolation of Lactic Acid Bacteria from Dangke. Fresh dangke samples made of cow and buffalo milk were purchased from Enrekang then stored in refrigerator until used for further processing. The dangke samples were weighed aseptically as 1 gram then crushed in a sterile mortar. Around 9 ml sterile distilled water was added, mixed thoroughly then prepared into dilution series. Dangke suspensions were inoculated onto de Man, Rogosa & Sharpe (MRS) Agar (Merck®) added with 1% CaCO3 using streak method then incubated for 5 x 24 h at 37 °C. The later growing colonies observed include: the shape, color, size and surface of the colony. Single colony that exhibited a clear zone the culture were transferred to MRSA added with 1% CaCO3 medium using quadrant streak method then incubated at 37 °C for 3-5 days. The same procedure was repeated until pure colony obtained then transferred to the MRSA slant. Pure isolates obtained were subjected into Gram staining, catalase test and 16s rRNA gene analysis to identify the strain.

2.1.2. Isolation of Lactic Acid Bacteria from Dangke. DNA extraction was performed following the protocol of Presto™ Mini GDNA Bacteria Kit for Gram positive (Geneaid®). PCR was carried in reaction mixture consisted of 12 μl of 27F (5'- AGAGTTTGATCMTGGCTCAG -3') Primer Forward and Reverse Primer 1492R (5'- TACGGYTACCTTGTTACGACTT -3'), 1.25μl 2G KAPA 2G Fast Ready Mix + Dye, 5μl of nuclease free water, and 5μl of DNA extract. Amplification was performed using a PCR device with an initial denaturation temperature of 95°C for 3 minutes, followed by 35 primary annealing cycles at 55°C for 10 seconds and extending 72°C for 30 seconds and post-extending for 1 minute. The PCR product was then visualized using electrophoresis on 1% agarose gel in TAE 1x buffer, 100 V power for 50 minutes then observed under UV light. PCR products were sent to PT. Genetika Indonesia for sequencing process. The sequencing results were analyzed using BLAST (http://www.ncbi.nlm.nih.gov/BLAST/). Multiple alignment was done by using Clustal W program. Gene analysis was done by reconstructing phylogenetic trees and comparing kinship between microorganisms using combination of MEGA 6 Program with Neighbor-Joining plot with bootstraps 1000x.

2.2. Production and Determination of Antimicrobial Activity of Crude Bacteriocin

LAB isolates were grown in de Man, Rogosa & Sharpe (MRS) Broth (Merck®) at 37 °C for 3 x 24 hours. After 72 hours, the culture medium was centrifuged at a speed of 3000 rpm for 15 minutes. The culture medium was then filtered using a 0.2 μm filter syringe. Bacteriocin precipitation was carried by adding ammonium sulphate with 80% saturation into the filtrate then the mixture was left overnight at 4°C. The partially purified bacteriocin was at a speed of 1500 rpm for 15 minutes then the filtrate
was discarded. Pellets contained bacteriocin were resuspended in phosphate buffer solution pH 7 and then dialyzed in buffer phosphate solution pH 7 solution for 48 hours.

Antimicrobial activity assay was carried in Mueller Hinton Agar (MHA) (Merck®) media using agar diffusion method. The culture of *S. aureus* and *E. coli* were rejuvenated in Nutrient Broth media for 18 hours then diluted according to the turbidity of McFarland Standard No. 0.5 (equivalent to 1.5x 10⁸ colony forming units). As much as 20μl of crude bacteriocin solution was pipetted into blank paper disk then affixed onto the media. Ampicillin disc was used as positive control while phosphate buffer pH 7.0 was used as negative control. Crude filtrates (pre-precipitation using ammonium sulphate) of both isolates were used as comparison. Media were incubated 37°C for 24 hours then inhibition zones were measured.

3. Result and Discussion

3.1. Isolation and Identification of the Isolates.

Two isolates were successfully obtained from cow and buffalo dangke. Both isolates were coded as B3B1 for isolates from buffalo dangke and as S2A1 for isolates from cow dangke. Colony morphology and Gram staining results are described in Table 1.

| Isolate | Source       | Form   | Margin | Color           | Elevation | Gram-staining                  | Catalase Test   |
|---------|--------------|--------|--------|-----------------|-----------|--------------------------------|-----------------|
| B3B1    | Buffalo dangke | Round  | Entire | Greyish white  | Convex    | Gram-positive, spherical      | Negative        |
| S2A1    | Cow dangke   | Round  | Entire | White           | Convex    | Gram positive, rod-shaped      | Negative        |

The sequence of isolate B3B1 showed high similarity up to 99% of the *Lactobacillus fermentum* strains while the sequence of isolate S2A1 showed high similarity up to 99% of the *Weisella confusa* strains. The results obtained show similarities with the results of the research done by Fatmawati et al. in 2017 [8], where they managed to conduct isolation and phenotypic identification of *L. plantarum* and *L. fermentum* from buffalo dangke. LAB in Dangke are originated from the milk used in the making and play a role in the maturation process of dangke [10]. The results of phenotypic identification of isolate B3B1 and S2A1 are presented in Table 2.

| Isolate | Closest Relatives          | Sequence length compared (bp) | Identity (%) | Accession No. |
|---------|----------------------------|-------------------------------|--------------|---------------|
| B3B1    | *Lactobacillus fermentum* strain NBRC 15885 | 1122                          | 99%          | NR_113335.1   |
|         | *Lactobacillus fermentum* strain CIP 102980 | 1122                          | 99%          | NR_104927.1   |
|         | *Lactobacillus gorlillae* strain KX01       | 1099                          | 98%          | NR_134066.1   |
| S2A1    | *Weisella confusa* JCM 1093                | 1024                          | 99%          | NR_113258.1   |
|         | *Weisella confusa* JCM 1093                | 1024                          | 99%          | NR_040816.1   |
|         | *Weisella cibaria* strain II-I-59          | 1016                          | 99%          | NR_036924.1   |

*L. fermentum* is one of LAB strain which often found in dairy products, several researchers have isolated *L. fermentum* from various source, e.g. mozzarella cheese [11], Mongolian traditional
fermented dairy products [12], infant feces [13] and chicken intestines [14]. Strains of *L. fermentum* are also found to have probiotic properties [8], ability to produce bacteriocin antibiotics [15] and cholesterol-reducing properties [16].

*Weisella confusa* strains belong to LAB group, with cow milk as their natural habitat. These strain not only possess probiotic properties but are also capable of producing prebiotic oligosaccharides [17]. *W. confusa* strains have been successfully isolated from fermented products such as boza, kimchi, sourdough, and nono—a fermented cow milk [17]. The sourcing of *W. confusa* bacteria from dangke in this study is the first time.

3.2. Production and Determination of Antimicrobial Activity of Crude Bacteriocin

Bacteriocin has been known for its antimicrobial properties, though it differs from traditional antibiotic due to its relatively narrow killing spectrum and selective toxicity towards bacteria closely related to its producer [4]. Purification of bacteriocin can be done by various methods. One conventional method commonly used for bacteriocin purification is precipitation with saturated ammonium sulphate, followed by dialysis [18].

Crude bacteriocins obtained from dialysis of ammonium sulphate precipitation of both strains in this study were found to freely dissolved in phosphate buffer pH 7.0 and had the characteristics of a clear orange solution for the S2A1 strain and clear solution in the B3B1 strain. The antimicrobial activity of crude bacteriocin against pathogen bacteria are shown in Table 3.

| Samples  | *Escherichia coli* Inhibition Zone After Re-incubation | *Staphylococcus aureus* Inhibition Zone After Re-incubation |
|----------|--------------------------------------------------------|-------------------------------------------------------------|
| S2A1 CF  | 10.35 ± 3.01 +                                        | 8.70 ± 2.10 +                                               |
| S2A1 CB  | 2.64 ± 4.57 -                                         | 5.36 ± 4.66 -                                              |
| B3B1 CF  | 8.57 ± 0.98 +                                         | 8.13 ± 0.57 +                                              |
| B3B1 CB  | 0                                                     | 11.34 ± 2.97 +                                             |
| PB       | 4.83 ± 4.22 -                                         | 5.30 ± 4.59 -                                              |
| Ampicillin | 16.53 ± 0.86 +                                       | 40.49 ± 3.34 +                                             |

CF = crude filtrate, CB = crude bacteriocin, PB = phosphate buffer pH 7.0, ( - ) zone disappear, (+) zone stay

Crude bacteriocin produced by isolate B3B1 was found to selectively active against *S. aureus* (narrow-spectrum activity). Thus, its activity was considered as intermediate (diameter 10-13 mm) [19]. Bacteriocin produced by Gram-positive bacteria usually show narrow-spectrum activity, they are likely to kill bacteria from the same ecological niche [20]. Crude bacteriocin of isolate B3B1 also was found to have bactericidal action, proven by a clear, permanent zone after re-incubation for 24 hours. Mechanism of bactericidal action of bacteriocin are proposed as: (1) formation of ion channel in cytoplasmic membrane, (2) display of nuclease activity, (3) induction of cell-autolysis, (4) interaction with amphipathic peptides which results in ion leakage that lead to cell death [20].

Limitation of this study was the lack of optimization in the production and purification process which greatly influenced the antimicrobial activity of bacteriocin. Thus, optimization in production and purification are suggested for further study.

4. Conclusion

Based on the result, isolate B3B1 identified as *Lactobacillus fermentum* from buffalo dangke has potential in producing bacteriocin. Further studies of production, optimization, characterization and screening of other bioactivity of the bacteriocin are suggested.
Acknowledgements
This study was financially supported by Universitas Hasanuddin, through the 2018 “Penelitian Dosen Pemula” Grant. We would also thank Haslia, S.Si., and the staffs of Science Building, Faculty of Mathematic and Natural Sciences, Hasanuddin University for their support in the study.

References
[1] Drider D, Bendali F, Naghmouchi K and Chikindas M L 2016 Bacteriocins: Not Only Antibacterial Agents Probiotics Antimicrob. Proteins 8 177–82
[2] López-Cuellar M del R, Rodríguez-Hernández A I and Chavarría-Hernández N 2016 LAB bacteriocin applications in the last decade Biotechnol. Biotechnol. Equip. 30 1039–50
[3] Dobson A, Cotter P D, Paul Ross R and Hill C 2012 Bacteriocin production: A probiotic trait? Appl. Environ. Microbiol. 78 1–6
[4] Riley M A and Wertz J E 2002 Bacteriocin diversity: Ecological and evolutionary perspectives Biochimie 84 357–64
[5] Mokoena M P 2017 Lactic acid bacteria and their bacteriocins: Classification, biosynthesis and applications against uropathogens: A mini-review Molecules 22
[6] Gülüç M, Karadayı M and Barış Ö 2013 Bacteriocins: Promising Natural Antimicrobials Microb. Pathog. Strateg. Comb. Chem. Sci. Technol. Educ. Vol. 2 1016–27
[7] Bryan C A O, Pak D, Crandall P G, Lee S O and Ricke S C 2013 The Role of Prebiotics and Probiotics in Human Health J. Probiotics Heal. 01 1–8
[8] Nur F, Hatta M, Natzir R and Djide M N 2017 Isolation of Lactic Acid Bacteria as a Potential Probiotic in Dangke, a Traditional Food from Enrekang, Indonesia Int. J. Sci. Basic Appl. Res. Int. J. Sci. Basic Appl. Res. 35 19–27
[9] Surono I S 2015 Traditional Indonesian dairy foods Asia Pac. J. Clin. Nutr. 24 S26–30
[10] Malaka R, Hatta W and Baco S 2017 Evaluation of using edible coating and ripening on dangke, a traditional cheese of Indonesia Food Res. 1 51–6
[11] de Souza B M S, Borgonovi T F, Casarotti S N, Todorov S D and Penna A L B 2019 Lactobacillus casei and Lactobacillus fermentum Strains Isolated from Mozzarella Cheese: Probiotic Potential, Safety, Acidifying Kinetic Parameters and Viability under Gastrointestinal Tract Conditions Probiotics Antimicrob. Proteins 11 382–96
[12] Bao Y, Zhang Y, Zhang Y, Liu Y, Wang S, Dong X, Wang Y and Zhang H 2010 Screening of potential probiotic properties of Lactobacillus fermentum isolated from traditional dairy products Food Control 21 695–701
[13] Archer A C and Halami P M 2015 Probiotic attributes of Lactobacillus fermentum isolated from human feces and dairy products Appl. Microbiol. Biotechnol. 99 8113–23
[14] Gusils C, Pérez Chaia A, González S and Oliver G 1999 Lactobacilli isolated from chicken intestines: Potential use as probiotics J. Food Prot. 62 252–6
[15] Riazi S, Nawaz S K and Hasnain S 2010 Bacteriocins produced by L. fermentum and L. acidophilus can inhibit cephalosporin resistant E.coli Brazilian J. Microbiol. 41 643–8
[16] Burhan H, Priyambada S A, Taufik E and Arief I I 2017 Potential of lactic acid bacteria isolated from Dangke and Indonesian beef as hypocholesterolaemic agent Media Peternak. 40 136–42
[17] Fusco V, Quero G M, Cho G S, Kabisch J, Meske D, Neve H, Bockelmann W and Franz C M A P 2015 The genus Weissella: Taxonomy, ecology and biotechnological potential Front. Microbiol. 6 155
[18] De Vuyst L and Leroy F 2007 Bacteriocins from lactic acid bacteria: Production, purification, and food applications Journal of Molecular Microbiology and Biotechnology vol 13 pp 194–9
[19] Venkadesan D and Sumathi V 2015 Screening of lactic acid bacteria for their antibacterial activity against milk borne pathogens undefined
[20] Jack R W, Tagg J R and Ray B 1995 Bacteriocins of gram-positive bacteria Microbiol. Rev. 59 171–200