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OPEN ACCESS Pakistan Journal of Nutrition ISSN 1680-5194 DOI: 10.3923/pjn.2017.645.650 Research Article Characterization of the Antimicrobial Activity of Lactic Acid Bacteria Isolated from Buffalo Milk in West Sumatera (Indonesia) Against Listeria monocytogenes 1Sri Melia, 1Endang Purwati, 1Yuherman, 1Jaswandi, 1Salam N. Aritonang and 2Mangatas Silaen 1Department of Animal Science, Andalas University, West Sumatra, Indonesia 2Department of Obstetrics and Gynecology, Medical Faculty of Prima Indonesia University, North Sumatra, Indonesia Abstract Background and Objective: Listeria monocytogenes is an important pathogenic bacteria in various cases of poisoning in the food industry due to its ability to grow in cold temperatures and to survive in freezing temperatures. Lactic acid bacteria have important probiotic attributes including their antimicrobial effect against this pathogen. Therefore, this study aimed to isolate lactic acid bacteria from buffalo milk and characterize its antimicrobial activity against Listeria monocytogenes. Materials and Methods: Buffalo milk was collected from four districts in West Sumatera, Indonesia and its composition analysed. A total of 88 lactic acid bacteria strains were isolated and grown at De Man Rogosa Sharpe Agar (MRSA). The strains were identified based on morphology (shape, size and colour) and their biochemical characteristics (catalase test and the fermentation type) and then screened for antimicrobial activity against L. monocytogenes. The species were further identified based on 16S rRNA gene sequence analysis. Results: As a result of isolation and identification, 19 strains of lactic acid bacteria were screened against L. monocytogenes, but only three isolates (A 3.2, A 3.3 and TD 7.2) showed high inhibition against L. monocytogenes. They were identified using 16S rRNA gene sequence analysis. Conclusion: The BLAST results of the identification procedure showed that the isolated bacteria from buffalo milk belonged to Lactobacillus fermentum strain L 23 (A 3.3), Lactobacillus fermentum strain 6704 (TD 7.2) and Lactobacillus oris strain J-1 (A 3.2). Key words: Lactic acid bacteria, buffalo milk, antimicrobial activity, 16S rRNA, inhibition zone and Listeria monocytogenes Received: April 17, 2017 Accepted: June 05, 2017 Published: July 15, 2017 Citation: Sri Melia, Endang Purwati, Yuherman, Jaswandi, Salam N. Aritonang and Mangatas Silaen, 2017. Characterization of the antimicrobial activity of lactic acid bacteria isolated from buffalo milk in West

Sumatera (Indonesia) against Listeria monocytogenes. Pak. J. Nutr., 16: 645-650. Corresponding Author: Sri Melia, Department of Animal Science, Andalas University, West Sumatra, Indonesia Tel: +62 8126761782 Copyright: © 2017 Sri Melia et al. This is an open access article distributed under the terms of the creative commons attribution License, which permits unrestricted use, distribution and reproduction in any medium, provided the original author and source are credited. 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. INTRODUCTION Buffalo (Bulabus bulabis) are domesticated ruminants that could be an alternative milk source. Due to its high fat and protein contents, buffalo milk could be used to produce cheese, yoghurt and ice cream and thus make a significant contribution to the dairy industry and its specific sensory properties might contribute to increasing the demand for various milk products. Buffalo milk is composed of 84.25 g kgG1 fat, 94.80 g kgG1 non-fat solids, 39.68 g kgG1 protein, 48 g kgG1 lactose, 7.13 g kgG1 ash, 0164% acid and 826.60 g kgG1 water and it has a pH of 6.371. Due to its lactose content, buffalo milk has the potential to grow lactic acid bacteria. Fresh milk might contain various types of microorganisms such as yeasts, moulds and bacteria, among which Lactic Acid Bacteria (LAB) are specifically recognized for producing lactic acid by fermenting milk sugar. Thus, lactic acid bacteria are presumed to be the most dominant bacteria in fresh milk, which may cause the pH of the milk to decrease due to their metabolism and thus provide a special environment that could prevent the proper growth of pathogenic bacteria2. As a result, lactic acid bacteria are classified as probiotic, particularly because they are antimicrobial, stomach-acid tolerant and safe to use3 but bacteria classified as probiotic should also have the ability to produce antimicrobial substances that can suppress the growth of pathogenic enteric bacteria. Such substances include organic acids, hydrogen peroxide, diacetyl and bacteriocin4. In the last decade, Listeria monocytogenes has become an important pathogenic bacteria that has been implicated in various poisoning cases related to the food industry due to its ability to grow at cold temperatures and survive freezing temperatures. Listeria monocytogenes is a gram-positive, spore- forming, cocci-shaped and intracellular pathogenic-type bacteria that can be found in monocytes and neutrophils5. Historically, this bacteria was classified in the genus Listerella, but in 1940, the generic name of this human and animal pathogen was changed to Listeria6. The optimum temperature for the growth of L. monocytogenes is 35-37EC but it can also grow between 1-5EC (psychropilic temperature) and it is also resistant to Pasteurization (72EC for 15 sec) and can survive at a pH range of 4.3-9.47. This research was conducted to assess the antimicrobial activity of lactic acid bacteria isolated from buffalo milk against L. monocytogenes. MATERIALS AND METHODS Materials: Buffalo milk was collected from four districts in West Sumatera, Indonesia (50 Kota, Agam, Tanah Datar and Solok). Samples were collected using sterile bottles and kept at a low temperature (8-10EC) during transport and prior to analysis. Methods Chemical composition: Chemical composition was determined following standard AOAC procedures8. Isolation and identification of lactic acid bacteria: A total of 88 isolates were obtained: 22 isolates from 50 Kota, 21 isolates from Agam, 25 isolates from Tanah Datar and 20 isolates from Solok. LAB strains were cultured in De Man Rogosa Sharpe broth (Merck, Germany) and spread on sterile MRS agar (Merck, Germany) plates, which were incubated at 37EC in anaerobic conditions for 48 h. The morphologically distinctive and well-strained colonies were selected by streaking to obtain pure colonies and then transferred to new MRS agar plates. Then, the selected colonies, which were positively proven to be catalase-negative and gram-positive were inoculated on new media for identification9. The morphological characteristics, particularly shape, colour and size, of the LAB were observed10 and Gram staining11, catalase testing12 and fermentation typing were conducted for biochemical evaluation13. Antimicrobial activity: To determine the antimicrobial effects of the selected LAB against L. monocytogenes (EP01), the agar-well diffusion method was used according to previous researches14,15 and a calliper was used to subsequently measure the obtained inhibition zone. The LAB strains were classified as bacteriocin producers when the wells formed an inhibitory zone16 and the clear area around the test wells was used to indicate inhibitory activity17. Therefore, the diameters (mm) of these zones were measured and recorded. Identification using 16S rRNA: The lactic acid bacteria species were further identified based on 16S rRNA gene sequence analysis. Genomic DNA from each strain was first extracted using the Extrap Soil DNA Kit Plus Ver. 2 and the 16S rRNA gene was amplified with the universal primers 27 F (5'-GAGTTTGATCCTGGCTAG-3'), 1525 R (5'-AGAAAGGAGGTGATCCAGCC-3'). The PCR amplification conditions were as follows: initial denaturation at 95EC for 5 min, 40 cycles of denaturation at 94EC for 45 s each, annealing at 56EC for 1 min, extension at 72EC for 1 min and 30 sec and final extension at 72EC for 7 min. The reaction mixtures were subsequently cooled to 4EC and the PCR products were analysed by agarose gel electrophoresis with 1% agarose. Subsequently, the PCR amplicons (approximately 1.5 kb) were purified with a Fast Gen Gel/PCR Extraction Kit (Nippon Genetics, Germany) according to manufacturer's instructions and the sequenced data were analysed and processed using BioEdit software. The sequences were compared with the sequences available in GenBank using BLAST (the Basic Local Alignment Search Tool) and all sequences were aligned using ClustalW (http://clustalW.ddbj.nig.ac.jp). RESULTS AND DISCUSSION The chemical composition of buffalo milk from some districts in West Sumatera are listed in Table 1, the buffalo milk was found to contain 7.22-7.83% protein, 7.18-7.88% fat and 80.62-81.03% moisture with a pH range from 6.06-6.39. The moisture and fat contents found in this study were similar to those of swamp buffalo milk (81% moisture and 7.0%) fat18. The pH range obtained in this study was lower than that reported for buffalo milk from the region of Cantal, France (pH 6.81)19. Total lactic acid bacteria in buffalo milk: As can be seen in Table 2, the total number of lactic acid bacteria from Agam and Tanah Datar was higher than that of 50 Kota and Solok. Moreover, the total LAB from this study was higher than that reported from Bulgarian Murrah buffalo (3.22×105 cmG3)20. The support Listeria monocytogenes, *Salmonella* Spp., and *Staphylococcus aureus*", Foodborne Pathogens and Disease, 02/2010">of LAB in dairy products microbiota can be viewed applicable Listeria monocytogenes, *Salmonella* Spp., and *Staphylococcus aureus*", Foodborne Pathogens and Disease, 02/2010">since these microorganisms are naturally show Listeria monocytogenes, *Salmonella* Spp., and *Staphylococcus aureus*", Foodborne Pathogens and Disease, 02/2010">in milking and processing enviroment, Listeria monocytogenes, *Salmonella* Spp., and *Staphylococcus aureus*", Foodborne Pathogens and Disease, 02/2010">facilitating the contamination of raw milk and processed products21. The LAB counts from raw milk were 8×105 C22. Lactic acid bacteria strains from buffalo milk: As can be seen in Table 3. Eighty-eight LAB strains from buffalo milk were evaluated in this study and 19 were screened for antimicrobial activity against Listeria monocytogenes. In general, such strains were rod-shaped, gram-positive, 1-4 mm in size, beige and catalase-negative. In addition, they did not show the ability to form CO2 and were thus classified as homofermentative; if bacteria can produce CO2, they are classified as heterofermentative. Homofermentative LAB have mostly been found in cow milk, cheese and Table 1: Composition of buffalo milk fermented milk23. Other reports have found homofermentative LAB strains in milk including Streptococcus cremoris and S. lactis24. Previous researchers successfully isolated Lactobacillus acidophilus, L. delbrueckii ssp. bulgaricus, Lactococcus lactis ssp. cremoris, L. lactis ssp. lactis and Streptococcus thermophilus25, Bifidobacterium spp. and Lactobacilli spp.9, Lactococcus lactis26. Lactic acid bacteria were isolated from various types of buffalo milk along with Lactobacillus plantarum, L. brevis, L. pentosus and Lactococcus lactis27,28. In addition, Lactococcus lactis, which can produce lactic acid, which is 57.61% of the lactic acid bacteria found in buffalo milk from North Sumatera along28. Antimicrobial activity against Listeria monocytogenes: As shown in Table 4 of the 19 screened LAB strains from buffalo milk, 3 were found to have higher inhibitory activity against L. monocytogenes, i.e., strain A 3.3 (19 mm) and A 3.2 (18 mm) from Agam and strain TD 7.2 (19 mm) from Tanah Datar. Martinez and de Martinis29,30 reported that Lactobacillus sakei, which produces bacteriocin, could decrease L. monocytogenes at 8EC. The bacteriocin of L. mesenteroides 11 partially inhibited L. monocytogenes at 8EC but at 15EC, it was unable to prevent the growth of the pathogen. Amezquita and Brashears31 found that the strain

identified as Pediococcus acidilactici was a possible bacteriocin producer with antilisterial activity. Some of the studied LAB have antimicrobial activity against L. monocytogenes and probiotic potential32,33; the LAB from Dadih Solok could inhibit L. monocytogenes given its 8-14-mm clear zone34. Molecular identification using 16S rRNA***: The isolates were molecularly identified by amplifying and sequencing the 16 S rRNA genes and comparing the results to the database of known 16S rRNA sequences. The BLAST results of the identification procedure showed that the isolated bacteria belonged to Lactobacillus fermentum strain L 23 (A 3.3), Lactobacillus fermentum strain 6704 (TD 7.2) and Lactobacillus oris strain J-1 (A 3.2). According to Pascual et al.35, Lactobacillus fermentum strain L 23 produces bacteriocins and it is heat-stable with a low-molecular-mass District Water (%) Fat (%) Protein (%) pH 50 Kota Agam Tanah datar Solok 81.44±1.17 7.18±0.09 7.22±0.39 6.06±0.04 80.62±0.93 7.34±0.50 7.57±0.21 6.39±0.25 80.48±1.12 7.73±0.22 7.23±0.76 6.10±0.02 81.03±1.91 7.88±0.98 7.83±0.19 6.08±0.03 Value represent Mean±SD, n = 3 Table 2: Total lactic acid bacteria of buffalo milk District Total LAB (1×106 CFU gG1) 50 Kota 3.0±0.820 Agam 190.0±30.51 Tanah datar 257.0±8.540 Solok 24.0±34.04 Table 3: Morphological and biochemical characteristic of the LAB Characteristic

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District Strain of LAB Morphology ---------- Form Gram stain Size Color Catalase test Biochemical ---------- Fermentatif type 50 Kota P 1.1 P 1.2 P 3.1 P 3.5 Rod + Rod + Rod + Rod + 1 mm 1 mm 2 mm 4 mm Cream - Cream - Cream - Cream - Homo fermentatif Homo fermentatif Homo fermentatif Homo fermentatif Agam A 1.2 A 3.1 A 3.2 A 3.3 A 3.4 Rod + Rod + Rod + Rod + Rod + 2 mm 2 mm 3 mm 3 mm 3 mm Cream - Cream - Cream - Cream - Cream - Homo fermentatif Hetero fermentatif Hetero fermentatif Homo fermentatif Homo fermentatif Tanah Datar TD 2.2 TD 2.3 TD 3.1 TD 3.2 TD 6.1 TD 6.3 TD 7.1 TD 7.2 Rod + Rod + Rod + Rod + Rod + Rod + Rod + Rod + 3 mm 1 mm 1 mm 1 mm 3 mm 3 mm 5 mm 1 mm Cream - Cream - Cream - Cream - Cream - Cream - Cream - Cream - Homo fermentatif Homo fermentatif Homo fermentatif Homo fermentatif Homo fermentatif Homo fermentatif Homo fermentatif Homo fermentatif Hetero fermentatif Solok S1.1 S1.3 Rod + Rod + 5 mm 3 mm Cream - Cream - Homo fermentatif Homo fermentatif Table 4: Antimicrobe activity of LAB strains against Listeria monocytogenes No. District Strain of LAB Inhibition zone (mm) 1 50 Kota P 1.1 17 2 P 1.2 17 3 P 3.1 12 4 P 3.5 14 5 Agam A 1.2 17 6 A 3.1 14 7 A 3.2 18 8 A 3.3 19 9 A 3.4 15 10 Tanah TD 2.2 17 11 Datar TD 2.3 15 12 TD 3.1 12 13 TD 3.2 15 14 TD 6.1 14 15 TD 6.3 15 16 TD 7.1 15 17 TD 7.2 19 18 Solok S1.1 11 19 S1.3 11 Value represent Mean±SD, n = 3 (<7000-Da) peptide. Yavuzdurmaz and Sebnem36 reported that Lactobacillus fermentum showed positive fermentation results for xylose, ribose, arabinose, melibiose, raffinose, galactose, maltose, sucrose, fructose and lactose; the fermentation result for Lactobacillus oris strain J-1 included ribose, arabinose, trehalose, melibiose, raffinose, galatose, maltose, sucrose, fructose and lactose. According to the classification, these biochemical characteristics indicate that the bacteriocin produced by Lactobacillus fermentum strain L 23 belongs to the class II lactic-acid bacterium bacteriocins37. Finally, the PCR products were sequenced and analysed using the basic local alignment search tool (BLAST, http://blast.ncbi.nlm.nih.gov/). CONCLUSION Isolation and identification resulted in 88 strains of LAB from buffalo milk, which were mostly characterized as rod-shaped, gram-positive, catalase-negative, homo-fermentative and heterofermentative. Of the isolates, only three (A 3.3, A 3.2 and TD 7.2) showed high inhibition against Listeria monocytogenes and were identified as Lactobacillus fermentum L23 (A 3.3), Lactobacillus fermentum 6704 (TD 7.2) and Lactobacillus oris strain J-1 (A 3.2). ACKNOWLEDGEMENTS This research was funded by the University of Andalas BOPTN Fund, Contract No. 869/XIII/A/Unand/2016 of April 22, 2016 and the Hibah Klaster Riset Guru Besar Universitas of Andalas, Contract No. 85/UN.16/HKRGB/LPPM/2016. We also acknowledge the Rector of the University of Andalas, the Chairman of LPPM, the Dean of the Faculty of Animal Science, the Counsellor, the Head of the Laboratory of Animal Product Technology and the Head of the Laboratory of Animal Biotechnology. REFERENCES 1. Khan, M.A.S., M.N. Islam and M.S.R. Siddiki, 2007. Physical and chemical composition of swamp and water buffalo milk: A comparative study. Ital. J. Anim. Sci., 6: 1067-1070. 2. Salminen, S. and A. von Wright, 2004. 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