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# Research Article
# Characterization of the Antimicrobial Activity of Lactic Acid Bacteria Isolated from Buffalo Milk in West Sumatera (Indonesia) Against *Listeria monocytogenes*

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## 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*

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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.

## 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 $kg^{-1}$ fat, 94.80 g $kg^{-1}$ non-fat solids, 39.68 g $kg^{-1}$ protein, 48 g $kg^{-1}$ lactose, 7.13 g $kg^{-1}$ ash, 0164% acid and 826.60 g $kg^{-1}$ water and it has a pH of 6.37[1]. 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 bacteria[2].

As a result, lactic acid bacteria are classified as probiotic, particularly because they are antimicrobial, stomach-acid tolerant and safe to use[3] 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 bacteriocin[4].

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 neutrophils[5]. Historically, this bacteria was classified in the genus *Listerella*, but in 1940, the generic name of this human and animal pathogen was changed to *Listeria*[6]. The optimum temperature for the growth of *L. monocytogenes* is 35-37°C but it can also grow between 1-5°C (psychropilic temperature) and it is also resistant to Pasteurization (72°C for 15 sec) and can survive at a pH range of 4.3-9.4[7].

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 (8-10°C) during transport and prior to analysis.

### Methods

**Chemical composition:** Chemical composition was determined following standard AOAC procedures[8].

**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 37°C 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 identification[9].

The morphological characteristics, particularly shape, colour and size, of the LAB were observed[10] and Gram staining[11], catalase testing[12] and fermentation typing were conducted for biochemical evaluation[13].

**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 researches[14,15] 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 zone[16] and the clear area around the test wells was used to indicate inhibitory activity[17]. 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 95°C for 5 min, 40 cycles of denaturation at 94°C for 45 s each, annealing at 56°C for 1 min, extension at 72°C for 1 min and 30 sec and final extension at 72°C for 7 min. The reaction mixtures were subsequently cooled to 4°C 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%) fat[18]. 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 ($3.22\times10^5$ $cm^{-3}$)[20].

The support of LAB in dairy products microbiota can be viewed applicable since these microorganisms are naturally show in milking and processing enviroment, facilitating the contamination of raw milk and processed products[21]. The LAB counts from raw milk were $8\times10^5$ C[22].

**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 $CO_2$ and were thus classified as homofermentative; if bacteria can produce $CO_2$, they are classified as heterofermentative. Homofermentative LAB have mostly been found in cow milk, cheese and fermented milk[23]. Other reports have found homofermentative LAB strains in milk including *Streptococcus cremoris* and *S. lactis*[24].

Previous researchers successfully isolated *Lactobacillus acidophilus, L. delbrueckii* ssp. *bulgaricus, Lactococcus lactis* ssp. *cremoris, L. lactis* ssp. *lactis* and *Streptococcus thermophilus*[25], *Bifidobacterium* spp. and *Lactobacilli* spp.[9], *Lactococcus lactis*[26]. Lactic acid bacteria were isolated from various types of buffalo milk along with *Lactobacillus plantarum, L. brevis, L. pentosus* and *Lactococcus lactis*[27,28]. 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 along[28].

**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 Martinis[29,30] reported that *Lactobacillus sakei*, which produces bacteriocin, could decrease *L. monocytogenes* at 8°C. The bacteriocin of *L. mesenteroides* 11 partially inhibited *L. monocytogenes* at 8°C but at 15°C, it was unable to prevent the growth of the pathogen. Amezquita and Brashears[31] 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 potential[32,33]; the LAB from Dadih Solok could inhibit *L. monocytogenes* given its 8-14-mm clear zone[34].

**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

Table 1: Composition of buffalo milk

| District | Water (%) | Fat (%) | Protein (%) | pH |
|---|---|---|---|---|
| 50 Kota | 81.44±1.17 | 7.18±0.09 | 7.22±0.39 | 6.06±0.04 |
| Agam | 80.62±0.93 | 7.34±0.50 | 7.57±0.21 | 6.39±0.25 |
| Tanah datar | 80.48±1.12 | 7.73±0.22 | 7.23±0.76 | 6.10±0.02 |
| Solok | 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 ($1\times10^6$ CFU $g^{-1}$) |
|---|---|
| 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 | | | | | |
|---|---|---|---|---|---|---|---|
| | | Morphology | | | | Biochemical | |
| District | Strain of LAB | Form | Gram stain | Size | Color | Catalase test | Fermentatif type |
| 50 Kota | P 1.1 | Rod | + | 1 mm | Cream | - | Homo fermentatif |
| | P 1.2 | Rod | + | 1 mm | Cream | - | Homo fermentatif |
| | P 3.1 | Rod | + | 2 mm | Cream | - | Homo fermentatif |
| | P 3.5 | Rod | + | 4 mm | Cream | - | Homo fermentatif |
| Agam | A 1.2 | Rod | + | 2 mm | Cream | - | Homo fermentatif |
| | A 3.1 | Rod | + | 2 mm | Cream | - | Hetero fermentatif |
| | A 3.2 | Rod | + | 3 mm | Cream | - | Hetero fermentatif |
| | A 3.3 | Rod | + | 3 mm | Cream | - | Homo fermentatif |
| | A 3.4 | Rod | + | 3 mm | Cream | - | Homo fermentatif |
| Tanah | TD 2.2 | Rod | + | 3 mm | Cream | - | Homo fermentatif |
| Datar | TD 2.3 | Rod | + | 1 mm | Cream | - | Homo fermentatif |
| | TD 3.1 | Rod | + | 1 mm | Cream | - | Homo fermentatif |
| | TD 3.2 | Rod | + | 1 mm | Cream | - | Homo fermentatif |
| | TD 6.1 | Rod | + | 3 mm | Cream | - | Homo fermentatif |
| | TD 6.3 | Rod | + | 3 mm | Cream | - | Homo fermentatif |
| | TD 7.1 | Rod | + | 5 mm | Cream | - | Homo fermentatif |
| | TD 7.2 | Rod | + | 1 mm | Cream | - | Hetero fermentatif |
| Solok | S1.1 | Rod | + | 5 mm | Cream | - | Homo fermentatif |
| | S1.3 | Rod | + | 3 mm | Cream | - | 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 Sebnem[36] 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 bacteriocins[37]. 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).

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