

SELECTION OF BUFFALO MILK LACTIC ACID BACTERIA WITH PROBIOTIC POTENTIAL

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ABSTRACT

Objective: The aim of this research was to isolate and identify lactic acid bacteria using 16S rRNA and evaluates their potential as probiotics.

Methods: The probiotic properties measured were resistance to low pH and to 0.3% and 0.5% bile salts, antimicrobial activity against pathogenic bacteria (*Escherichia coli* O157:H7 and *Staphylococcus aureus* ATCC 25923), antibiotic resistance, and hydrophobicity.

Results: The lactic acid bacteria with optimal probiotic properties were isolated from buffalo milk and identified from a sample from Agam district (BMA 3.3) which was classified using BLAST analysis as a strain of *Lactobacillus fermentum* (L23).

Conclusion: Buffalo milk from this part of West Sumatera contains a strain of *L. fermentum* with has good probiotic properties.

Keywords: Lactic acid bacteria, Probiotic, Buffalo milk, *Lactobacillus fermentum* strain L23.

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INTRODUCTION

Probiotics are living microorganisms that provide health-promoting effects for their host. Before a bacterium can be said to be probiotic, it must meet several criteria, including the ability: To survive in the presence of acids and bile salts, to produce antimicrobial compounds, and to colonize the intestines and resist antibiotics [1]. Common probiotics are lactic acid bacteria and bifidobacteria, which are isolated from fermented products, digestive systems, feces, and human breast milk. Researchers have started to look at other potential sources of probiotics including buffalo milk. Some probiotics have already been isolated from buffalo milk, [2] isolated *Lactobacillus acidophilus*, *Lactobacillus rhamnosus*, and *Bifidobacterium longum*, all potential probiotics, from buffalo milk from Karnataka, India, but little information is available regarding potential probiotic bacteria in buffalo milk in West Sumatera where the animals are common.

Buffalo milk plays a significant role in satisfying the nutritional demands of humans in a number of developing countries [3]. Buffalo milk has a rich complex nutritional profile. However, in West Sumatera, it is rarely consumed in its raw state. Here, milk from swamp buffalo (*Bubalus bubalis*) is fermented in a bamboo tube for 48 h to produce Dadih. Dadih production occurs in several different areas throughout West Sumatra. If proven to have probiotic properties, more extensive use of this local dish could be encouraged to improve the health and nutritional status of people in this area who tend to be resistant to adopting non-traditional foods.

There is good reason to believe that West Sumatera buffalo milk might be such a source of probiotics [2]. Isolated *L. acidophilus*, *L. rhamnosus*, and *B. longum* are potential probiotics, from buffalo milk from Karnataka, India [4]. Isolated lactic acid bacteria from buffalo milk in Islamabad, namely, *L. acidophilus*, *Lactobacillus delbrueckii* ssp. *bulgaricus*, *Lactococcus lactis* ssp. *cremoris*, *L. Lactis* ssp. *lactis*, and *Streptococcus thermophilus*. Similarly, Sharma *et al.* [5], also found *L. lactis* in buffalo milk in India [6], found strains of *Lactobacillus* spp., isolated from buffalo, goat, and cow milk, had potential as probiotics. Previous research on the content of lactic acid bacteria from buffalo milk from Pampangan North Sumatera has been conducted by Rizqiati *et al.* [7] who found several isolates of lactic acid bacteria, namely,

Lactobacillus brevis, *Lactobacillus paracasei*, *Lactobacillus penthouse*, *Lactobacillus planetarium*, and *L. lactis*.

However, the probiotic potential of lactic acid bacteria contained in buffalo milk in West Sumatera has yet to be studied.

METHODS

Lactic acid bacteria isolated from buffalo milk from several districts in West Sumatera were obtained and were coded according to the area it was sourced from. These were screened for acid resistance and five isolates from different areas with acid resistance above 50% were obtained, namely, those coded BMA 3.3 (Agam District), BMTD 7.2 (Tanah Datar District), BMP 1.1 (Limapuluh Kota District), BMS 1.1 (Solok District), and BMSJ 4.2 (Sijunjung District). The bacteria were then tested for probiotic properties after being grown in de Man, Rogosa, and Sharp (MRS) broth (Merck, Germany) for 24 h at 37°C.

Lactic acid bacteria resistance test against acid condition

Effect of acidic conditions (pH 2) was tested using MRS broth (Merck) regulated with HCl 1 N and MRS broth (Merck, pH 5.7) as controls. Both media were inoculated with 1% isolate, at 37°C for 90 min and for 180 min, and measured at A_{600nm} [8].

Lactic acid bacteria resistance against bile salts

Testing with bile salts was conducted to see if the bacteria would survive in the human small intestine. Any probiotic must survive several hours in this part of the digestive system before it reaches the large intestine where its presence provides the health benefits for the host. 1% isolates were inoculated into MRS broth containing 0.3% and 0.5% bile salts (Oxgall, Merck, Germany) for 5 h at 37°C. After 5 h, the bacterial populations were measured at A_{600nm} and compared to a control (without addition of bile salts). Results were recorded as the ratio of growth rate between the bile salts and controls at A_{600nm} [8,9].

Antibacterial activity test against pathogens

Modification of the well diffusion assay of Yang *et al.*, Ayeni *et al.* [10,11] was used to test the antibacterial activity against pathogens *Escherichia coli* O157:H7 and *Staphylococcus aureus* ATCC 25923. Cell-free supernatant derived from lactic acid bacteria that had been

grown in MRS broth for 24 h at 37°C, in anaerobic conditions and then centrifuged at 10,000 rpm for 5 min at 4°C. 50 mL of the supernatant was placed in a well on which pathogenic bacteria on MHA medium (Mueller Hinton Agar, Merck) were growing. The size of the inhibition zone indicating antibacterial activity of the isolate was measured after 24 h.

Antibiotic resistance test

Isolates of lactic acid bacteria were tested for resistance to antibiotics using the method of the Clinical and Laboratory Standards Institute [12]. Antibiotics used in this study were ampicillin (10 µg), chloramphenicol (30 µg), erythromycin (15 µg), penicillin (10 µg), and tetracycline (30 µg). These were placed on MRS agar plates (Merck) which already contained the lactic acid bacteria. The plates were then incubated anaerobically at 37°C, for 24 h. The inhibition zone diameter was measured. The results were categorized as resistance (R), intermediates (I), and sensitive (S) according to the standard inhibition diameter for each antibiotic.

Hydrophobicity test

Measurements to determine the cell-surface hydrophobicity can be conducted *in vitro* by measuring the percentage adherence of microbial cells to solvents such as xylene, toluene, or n-hexadecane [13]. Xylene, toluene, and n-hexadecane can be used because they are non-polar and their hydrophobic properties help interact with the hydrophobic microbial surfaces.

The hydrophobicity test was conducted using xylene to determine the adhesive properties of lactic acid bacteria isolates *in vitro* [14]. Lactic acid bacteria isolates were incubated (anaerobically) at 37°C for 18–22 h and centrifuged at 10,700 rpm for 5 min [15]. After that, the bacterial cells were washed twice with PBS pH 7 and OD 600 nm (A0) was read. A suspension of 3 mL lactic acid bacteria was mixed with 1 mL of xylene for 60 s, then incubated for 2 h at 37°C. The aqueous phase at the bottom was taken and OD 600 nm measured (A1). The resulting hydrophobicity is calculated by subtracting A1 divided by A0 from A0, then multiplying the result by 100% [16].

Identification of lactic acid bacteria using 16S rRNA sequencing

Lactic acid bacteria isolates were cultured in MRS broth at 37°C for 24 h. Isolation of genomic DNA was carried out using Extrap Soil DNA Kit Plus Ver.2. Polymerase chain reaction (PCR) amplification of the isolates' 16S rRNA using a 16S rRNA fragment gene of ~1.5 KB was conducted using universal primers 27 F:(5'-GAGTTTGATCCTGGCTAG-3') and 1525 R:(5'-AGAAAGGAGGTGATCCAGCC-3'). Initial denaturation at 95°C for 5 min with 25 cycles was followed by denaturation at 94°C for 1 min, then annealing at 56°C for 1 min, extension at 72°C for 1.5 min, and final extension at 72°C for 7 min. The resulting DNA was separated out using electrophoresis at 100 V for 21 min, using a 1% agarose in ×1 TAE buffer. Then, a gel documentation system was used to produce an image of the bands in the gel. Purification was conducted using a fast gene gel/PCR extraction kit (Nippon Genetics, Germany), and the resulting sequences analyzed using the BLAST program at the NCBI gene bank database that can be viewed at <http://blast.ncbi.nlm.nih.gov/Blast.cgi>. Sequence alignments were prepared using Bioedit application, and the phylogenetic tree was created with the MEGA application 6.

RESULTS AND DISCUSSION

Lactic acid bacteria resistance to acid conditions

Any effective probiotic must survive passage through the digestive tract so must be resistant to the acidic conditions of the stomach of pH of 1.5–3.5, which are caused by the secretion of gastric juice.

Fig. 1 shows the resistance of lactic acid bacteria to pH 2 (gastric acid pH) at 90 min and 180 min. The BMA 3.3 isolate, which was incubated at pH 2 and 90 min, showed resistance up to 95.018% after 90 min and 73.944% at 180 min. This decrease of 23% was quite small compared to other isolates. For example, BMS 1.1 experienced a 37% decrease indicating lower resistance to acid. This result for BMA 3.3 is even higher than the lactic acid bacteria isolated from milk and cattle dung samples by Jain *et al.* [13], which is regarded as a potential probiotic because it

can survive at pH 2 (56.93–80.88%) and pH 3 (61.44–81.25%). While the lactobacillus investigated by Singh *et al.* [17] showed a tolerance to acid at pH 2 (46.47–79.74%).

Lactic acid bacteria resistance against bile salts

Effective probiotics must also be able to thrive in the alkaline pH of the small intestine which is affected by bile salts secreted by the liver. Bile reduces the number of bacteria by destroying their cell walls. The concentration of bile salts in the human body is 0.3–0.5% [18–20], and several probiotic bacteria have been shown to survive these concentrations [21], found *Lactobacillus oris* HM168 isolated from breast milk, survives at 0.3% and 0.5% bile salt for 5 h. Likewise, the strain of lactic acid bacteria isolated from buffalo milk from Karnataka, India, was able to survive in concentrations of 0.3% and 0.5% bile salts, but this was not sustained at 1% [2]. The five isolates of lactic acid bacteria were tested for bile salt resistance of 0.3% and 0.5%, for 5 h.

Fig. 2 demonstrates that the highest resistance to bile salts at 0.3% is shown by the BMSJ 4.2 isolate, followed by BMS 1.1, BMA 3.3, BMP 1.1, and BMTD 7.2. However, at 0.5% bile salt, the BMA isolate 3.3 showed the highest resistance experiencing the lowest decrease of all isolates (13%). BMA 3.3's 5–26% decrease due to acid followed by a 35–40% decrease after exposure to bile salts falls within the criteria quoted by Bezkorovainy [22] that to be a useful probiotic in the human digestive system a bacterium's resistance to gastric acid and bile salts should be between 20% and 40%.

Antibacterial activity against pathogens

Effective probiotics must be antimicrobial that is they help to control pathogenic bacteria in the digestive tract. The ability of lactic acid bacteria to inhibit the growth of pathogenic bacteria is influenced by their ability to produce antimicrobial compounds such as bacteriocin [1] and organic acids. Bacteriocin actively attacks Gram-positive pathogenic bacteria such as *Listeria monocytogenes* and *S. aureus* and also some Gram-negative bacterial pathogens [23,24]. For example, *Lactobacillus pentosus* and *Lactobacillus plantarum* from

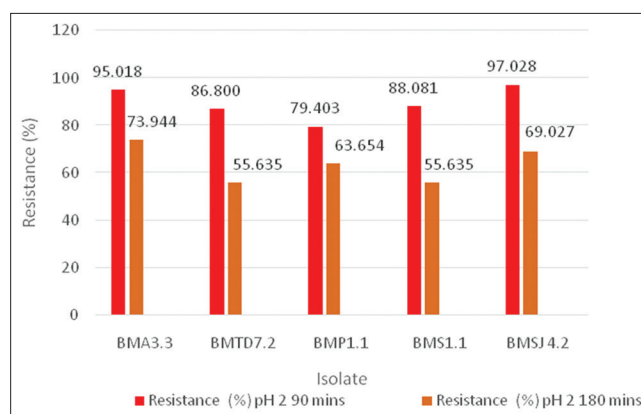


Fig. 1: Lactic acid bacteria resistance to acid conditions

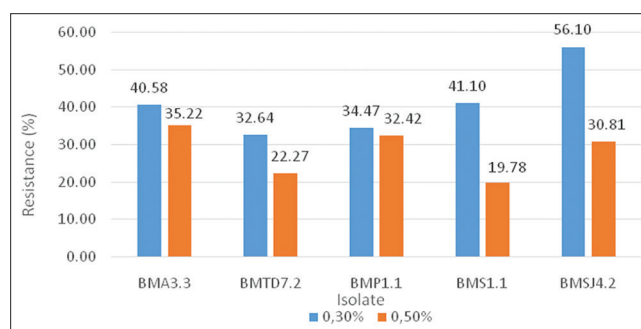


Fig. 2: Lactic acid bacteria resistance against bile salt

okara was found to have antimicrobial activity against *E. coli*, *S. aureus*, and *L. monocytogenes* [25].

Table 1 summarizes that all lactic acid bacteria isolates were capable of inhibiting the growth of pathogenic bacteria. The highest antibacterial activity against *E. coli* O157:H7 was demonstrated by BMA 3.3 and BMS 1.1. The highest antibacterial activity against *S. aureus* ATCC 25923 was from BMP 1.1. Overall, BMA 3.3 shows the most promising antibacterial properties against both common pathogens. The antibacterial activity of BMA 3.3 isolates against *S. aureus* ATCC 25923 and *E. coli* O157:H7 was lower than that of *Lactobacillus fermentum* isolated from buffalo milk by Jain et al. [13], which had higher antibacterial activity against *S. aureus* (20.34 ± 0.02 mm). However, the strain does show a significant protective effect against both *E. coli* and *S. aureus*.

Antibiotic resistance of lactic acid bacteria

The sensitivity of lactic acid bacteria to antibiotics can be seen in Table 2. All lactic acid bacteria isolates were resistant to ampicillin and sensitive to tetracycline and chloramphenicol, except BMP 1.1 and BMSJ 4.2 isolates which showed intermediate susceptibility to chloramphenicol. Apart from BMS 1.1, all isolates were resistant to both erythromycin and penicillin [26], also found that lactic acid bacteria they isolated were resistant to erythromycin and penicillin.

Hydrophobicity of lactic acid bacteria

Effective probiotics must be able to colonize the digestive tract to provide protection against pathogenic microbes. *Lactobacilli* with hydrophobic cell surfaces can do this as they attach to the host gastrointestinal tract.

According to Sánchez-Ortiz et al. [27], 30% is considered low hydrophobicity (adhesion to p-xylene), 30–60% is medium, and 60% is high hydrophobicity. Hydrophobicity percentages can be seen in Fig. 3. Medium hydrophobicity was measured for BMA 3.3 and BMTD 7.2. The highest hydrophobicity was shown by BMA 3.3 (55.492 ± 0.823). This value is lower than the results of the research of Tokath et al. [28] who found hydrophobicity percentages from *L. brevis* MF105 of 97.96%, *L. plantarum* MF265 of 82.41%, *L. brevis* MF949 of 67.29%, and *L. brevis* MF493 of 62.36%. These four isolates were categorized as probiotics.

Identification of lactic acid bacteria from 16S rRNA

Of the five isolates of lactic acid bacteria from buffalo milk from different regions, isolate BMA 3.3 was selected as being the most promising as it has the highest resistance to acid and bile salts, highest antimicrobial activity, good antibiotic resistance, and highest hydrophobicity. 16S rRNA sequence analysis identified this isolate as *L. fermentum* strain L2.3 (Figs. 4 and 5). Interestingly, this bacterial strain appears to be identical to one of the lactic acid bacteria that were isolated from buffalo milk in Mumbai [29]. *L. fermentum* L2.3 has also been found in the human vagina and found to be protective against *E. coli* [30]. Almost 48% of the lactic acid bacteria in West Sumatran buffalo milk were *L. fermentum* followed by *L. acidophilus* (34%), *L. viridescens* (8%), *L. brevis* (5%), and *L. gasseri* (4%). This

combination differs from that found in buffalo milk from Karnataka, India, by Shafakatullah and Chandra [2] who isolated potential probiotic lactic acid bacteria; *L. rhamnosus* and *B. longum* along with *L. acidophilus*.

CONCLUSION

The results identified one lactic acid bacteria isolated from a sample of swamp buffalo milk harvested in Agam district that showed high probiotic potential. Of all the isolates, BMA 3.3 had the best ability to survive at low pH, resist bile salts. It effectively inhibited the growth of pathogenic bacteria (*E. coli* O157: H7 and *S. aureus* ATCC 25923) and was resistant to three of the five most commonly used antibiotics and had medium hydrophobicity. This lactic acid bacterial strain was identified as *L. fermentum* strain L23. This isolate has potential as a natural addition in the production of fermented milk-based foods to enhance their health value.

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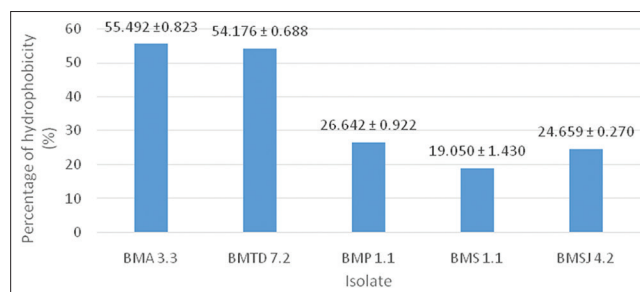


Fig. 3: Hydrophobicity percentage of lactic acid bacteria

Table 1: Antibacterial activity against pathogens

Isolate	Inhibition zone (mm)	
	<i>E. coli</i> O157:H7	<i>S. aureus</i> ATCC 25923
BMA 3.3	16±0.82	19±0.47
BMTD 7.2	15±0.82	15±0.94
BMP 1.1	12±0.00	18±0.82
BMS 1.1	16±0.82	16±0.47
BMSJ 4.2	9±0.94	14±0.47

The value is expressed as the mean±standard deviation; n=3, *E. coli*: *Escherichia coli*, *S. aureus*: *Staphylococcus aureus*

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TCGAACGCGTTGGCCCAATTGATTGATGGTGCCTTGCACCTGATTGATTTGGTCGCCAACGAGTGGCGG
ACGGGTGAGTAACACGTAGGTAACCTGCCAGAAAGCGGGGACAACTTTGGAAACAGATGCTAATA
CCGCATAACAACGTTGTCGCATGAACAACGCTTAAAAGATGGCTTCTCGTATCACTTCTGGATGGA
CCTGCGGTGCATTAGCTTGTGGTGGGGTAACGGCTACCAAGGCGATGATGCATAGCCGAGTTGAGA
GACTGATCGGCCAATGGGACTGAGACACGGCCATACTCCTACGGGAGGCAGCAGTAGGGAATCT
TCCACAATGGGCGCAAGCCTGATGGAGCAACACCGCGTGAGTGAAGAAGGGTTTCGGCTCGTAAAGC
TCTGTTGTTAAAGAAGAACACGATGAGAGTAACCTGTCATACGTTGACGGTATTTAACAGAAAAGTC
ACGGCTAACTACGTGCCAGCAGCCGCGTAATACGTAGGTGGCAAGCGTTATCCGGATTTATTGGGCG
TAAAGAGAGTGCAGGCGGTTTTCTAAGTCTGATGTGAAAGCCTTCGGCTTAACGGAGAAGTGCATCG
GAAACTGGATAACTGAGTGCAGAAGAGGGTAGTGAACCTCATGTGTAGCGGTGAATGCGTAGAT
ATATGGAAAGAACACAGTGGCGAAGGCGGCTACCTGGTCTGCAACTGACGCTGAGACTCGAAAGCAT
GGGTAGCGAACAGGATTAGATACCCTGGTAGTCCATGCCGTAACGATGAGTGTAGGTGTTGGAGG
GTTTCCGCCCTTCAGTGCCGGAGCT
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Fig. 4: Nucleotide sequence of *Lactobacillus fermentum* strain L23

Table 2: Sensitivity of lactic acid bacteria isolates to different antibiotics (diameter of the inhibition zone in mm)

Isolate	Antibiotics				
	Ampicillin	Chloramphenicol	Erythromycin	Penicillin	Tetracycline
BMA 3.3	5±0.82 (R)	21±0.47 (S)	12±1.25 (R)	8±1.70 (R)	24±2.62 (S)
BMTD 7.2	3±0.82 (R)	20±0.94 (S)	0 (R)	0 (R)	26±2.05 (S)
BMP 1.1	3±0.94 (R)	14±1.70 (I)	3±0.82 (R)	4±0.47 (R)	22±1.41 (S)
BMS 1.1	12±0.47 (R)	27±0.47 (S)	24±1.25 (S)	16±1.41 (S)	25±1.41 (S)
BMSJ 4.2	0 (R)	18±0.94 (I)	0 (R)	0 (R)	28±0.94 (S)

R: Resistant, I: Intermediate, S: Sensitive. The value is expressed as the mean ±, standard deviation; n=3

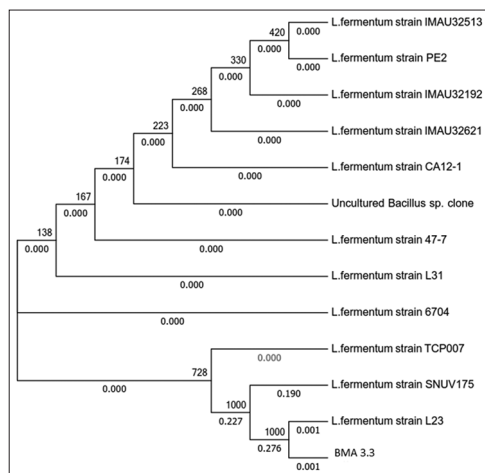


Fig. 5: Phylogenetic tree of *Lactobacillus fermentum* strain L23

AUTHOR'S CONTRIBUTION

Sri Melia designed, conducted, and wrote up the search, and Yuherman, Jaswandi, and Endang Purwati provided guidance and helped with manuscript revision.

CONFLICT OF INTERESTS

There is no conflict of interest.

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