Isolation and Screening of Lactic Acid Bacteria from Sumbawa Buffalo Milk (*Bubalus bubalis*) as Potential Starter Cultures

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Abstract

The Sumbawa buffalo (*Bubalus bubalis*) is one of the ruminant livestock in Indonesia that not only contributes to fulfilling meat requirements but also milk. Besides containing nutrients that are very beneficial for human health, buffalo milk is also a potential source of lactic acid bacteria (LAB) with technological and functional properties. Lactic acid bacteria have been utilized as starter cultures in various fermented products. This study aimed to isolate LAB from Sumbawa buffalo milk and to identify the potential isolate as a starter culture. The screening of LAB as a starter culture was based on some technological properties, including proteolytic activity, lipolytic activity, exopolysaccharide (EPS) production, antibacterial activity, antibiotic sensitivity, hemolytic activity, and acidification activity. Data were analyzed statistically using one-way ANOVA and Tukey's post-hoc test at a 5% significance level. A total of 21 isolates were isolated from fresh buffalo milk, with a LAB total was $4.7x10^5$ CFU.mL^{-1}. All the isolates were characterized as Gram-positive with cocci-shaped. The SA8 isolate was selected as the most potential candidate as a starter culture because it has fulfilled the criteria such as the highest proteolytic activity, the lowest lipolytic activity, producing EPS, potential antagonistic activity against *Bacillus cereus*, *Escherichia coli*, and *Salmonella* Typhi, and sensitivity to cefazolin, intermediate to erythromycin and cinoxacin, non-pathogen, as well as the most rapid acidification activity. The SA8 isolate was identified as *Enterococcus lactis* with a similarity level of 99.99% towards strain BT159. This indigenous LAB was a potential starter culture of Sumbawa fermented buffalo milk to increase the diversification of products derived from buffalo milk.

Keywords: *Enterococcus lactis*, lactic acid bacteria, technological properties, starter culture, Sumbawa buffalo milk.

INTRODUCTION

Milk is a commodity produced by livestock that is important for fulfilling nutritional needs to improve public health. One of the main livestock supply areas for several regions in Indonesia is West Nusa Tenggara Province. This province is ideal for livestock farming due to the availability of grazing land that provides feed for the needs of ruminant livestock, for example, buffalos, cattle, and horses [1]. Based on the Central Bureau of Statistics data in 2019, the three provinces with the most buffalo farming sectors are East Nusa Tenggara, West Nusa Tenggara, and North Sumatra. In West Nusa Tenggara, the Sumbawa buffalo has different morphological and genetic characteristics than mud buffalo or other local buffalo clumps. Sumbawa buffalo has distributed in the Sumbawa Island, West Nusa Tenggara Province. The buffalo milk produced by farmers in Sumbawa averages 1-3 liters per cows per day. This milk production is quite high compared to the average buffalo milk production in general [2].

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Buffalo milk has some benefits for human health. It contains bioprotective, immunoglobulins, lactoferrins, lysozymes, lactoperoxidase, and bifidogenic [3]. Buffalo milk has prominent nutritional content, such as fat, protein, lactose, and minerals (calcium, iron, magnesium, and phosphorus). It has cholesterol levels almost twice the content of conjugated linoleic acid. Conjugated linoleic acid is one of the other forms of essential fats that human needs [4]. Monounsaturated fatty acids contained in buffalo milk can provide antioxidant activity [5], improve bone health and reduce the risk of osteoporosis [6], and maintain blood pressure levels [7]. Buffalo milk also has potential economic value through fermentation for producing fermented milk products manufactured either traditionally or industrially [8]. In addition, the fermentation process can reduce milk spoilage due to the role of lactic acid bacteria (LAB) in inhibiting the growth of undesirable microbes.

Lactic acid bacteria are characterized by a Gram-positive, catalase-negative, with a rod or cocci-shaped [9]. During fermentation, glucose can be converted by LAB into lactic acid [10], and some LAB probiotics are beneficial to maintain intestinal microflora balance [11]. The use of indigenous LAB, native microflora that inhabit

certain habitats, as a starter culture for producing fermented milk products can increase the fermented product quality in terms of aroma, taste, and texture [12] and can inhibit the growth of pathogenic bacteria [13]. Starter cultures are defined as microbes used to start, direct, and accelerate the fermentation process [14]. The milk fermentation, with the addition of starter cultures, can also increase nutritional values through increasing nutritional bioavailability and the production of bioactive substances with functional properties [15].

Indigenous bacteria are believed to be more effective when used as a starter culture in fermentation. Some criteria of starter cultures in producing fermented products, especially yogurt, are high proteolytic activity, low lipolytic activity, producing exopolysaccharide (EPS), high antibacterial activity, high sensitivity to antibiotics, non-pathogen, and high acidification activity. The LAB exploration of Sumbawa buffalo milk and its use as a starter culture has not been widely reported yet. Therefore, this study aimed to isolate LAB from Sumbawa buffalo milk and to identify the potential isolate as the starter culture candidates.

MATERIAL AND METHOD

Sampling Collection of Sumbawa Buffalo Milk

The determination of the sampling location was carried out by purposive sampling by taking into account the high level of the buffalo population. Buffalo milk samples were taken from two different locations, namely Srangen Hamlet (Sample S) in Bugis Village and Tanakakan Hamlet (Sample T) in Menala Village, each of which is in Taliwang District, West Sumbawa Regency, West Nusa Tenggara. The buffalo milk samples obtained directly from farmers were put into sterile bottles, and then stored in a cool box (± 4°C) during transportation from the sampling site to the laboratory.

Nutritional Content Analysis

The nutritional content (proximate) analysis was carried out following the AOAC procedure [16]. The parameters measured include water content, ash content, fat content, and protein content. The proximate content provides information about the nutritional values of the Sumbawa buffalo milk.

Isolation of LAB

The LAB isolation used a selective medium of MRS and M17 agar containing 1% $CaCO_3$ [9]. The serial dilution of the sample was carried out in 9 mL of sterile saline water (NaCl 0.85%). A total of 100 µL of the samples was inoculated in the media using a spread plate technique. The cultures were incubated in aerobic conditions at 37°C for 48 h. The colonies with a clear zone were selected. The total colony of LAB was calculated based on the total plate count (TPC) method. Furthermore, isolates were characterized using Gram staining and catalase tests to confirm the LAB characteristics. The purified LAB isolates were maintained in MRS agar slant for stock cultures.

Proteolytic Activity Test

The proteolytic activity was conducted using a 1% skim milk agar medium, which was enriched with 0.05% NaCl, 0.1% yeast extract, 0.2% tripton, 0.01% $CaCl_2$, and 1.5% Bacto agar [17]. The 24-h LAB cultures were equalized using a spectrophotometer with a wavelength of 600 nm, and then 50 µL of the cultures were dripped onto a blank disk (Oxoid, UK). The disks were then placed on the surface of skim milk agar and incubated at 37°C for 24 h. A negative control used was media without cultures. Proteolytic activity was observed, indicated by the presence of clear zones around the blank disks, and it is measured in millimeters and calculated using Formula 1. The isolates with high proteolytic activity were selected for the subsequent assay.

$$\mathrm{DCZ} = \mathrm{DT\text{-}DC} \ \ldots\ldots\ldots\ldots\ldots\ldots\ldots (1)$$

Description :

DCZ : Diameter of the clear zone (mm)
DT : Diameter of total (mm)
DC : Diameter of colony (mm)

Lipolytic Activity Test

The lipolytic activity test was conducted by inoculating 50 µL of the selected LAB cultures onto a blank disk (Oxoid, UK) and placed on the Sierra agar media containing 1 % peptone, 0.5% NaCl, 0.01% $CaCl_2.2H_2O$, 1.5% Bacto agar, 1% Tween 80, and 0.01% methyl red. Subsequently, the cultures were incubated at 37°C for 96 h [18]. A negative control used was media without cultures. The lipolytic activity was observed by the appearance of clear zones around the blank disks, and it is measured in millimeters and calculated using Formula 1. The isolates with the absence or low lipolytic activity were selected for the subsequent assay.

Exopolysaccharide Production Test

Exopolysaccharide (EPS) production test was carried out by culturing the LAB isolates in MRS agar supplemented with 5% sucrose and incubated at 37°C for 24-48 h [19]. The positive results of EPS production were indicated

qualitatively by forming thick slime or mucoid colonies. The isolates showing this character were then selected for the next assay.

Antibacterial Activity Test

The antibacterial activity test was performed using the disk-diffusion agar [17]. The indicator bacteria used were *Escherichia coli*, *Staphylococcus aureus*, *Bacillus cereus*, and *Salmonella* Typhi. These indicator bacteria were selected to represent the enteric pathogens and are usually found as contaminant bacteria in milk. The selected LAB cultures with a density of 10^7 CFU.mL^{-1} were centrifuged at 10,000 x g at 4°C for 10 mins. The cell-free supernatant (CFS) was taken, and the pH was adjusted using NaOH 4 M to pH 7. The CFS was filter-sterilized using a 0.22 µm milipore membrane. The filter-sterilized CFS (50 µL) was dripped onto a blank disk (Oxoid, UK). The disks were then put onto nutrient agar containing 100 µL of indicator bacteria with a cell density of 10^6 CFU.mL^{-1} and then incubated at 37°C for 24 h. The inhibition zones around the disks were in millimeters and calculated using Formula 1. A negative control used was media without cultures, while streptomycin 10 µg was used as a positive control.

Antibiotic Sensitivity Test

The antibiotic sensitivity test was conducted using the disk-diffusion agar technique referring to James [20]. The antibiotics used were Kanamycin 30 µg, Erythromycin 15 µg, Cinoxacin 100 µg, and Cefazolin 30 µg. After the selected LAB cultures achieved 10^8 CFU.mL^{-1}, 50 µL of the LAB cultures were spread onto the MRS agar using a sterile cotton swab, and the antibiotic disks were placed on the surface of the agar media and incubated at 37°C for 24 h [20]. The disk dripped with MRS broth media was used as a negative control. The inhibition zones around the disks were in millimeters and calculated using Formula 2. The sensitivity to antibiotics was categorized as shown in Table 1 [20].

$$\mathrm{DCZ = DT\text{-}DC} \ldots\ldots\ldots\ldots (2)$$

Description :

DCZ : Diameter of the clear zone (mm)
DT : Diameter of total (mm)
DC : Diameter of disk (mm)

Table 1. Criteria for sensitivity to antibiotics

Antibiotics	Diameter of inhibition zone (mm)		
	Resistant (≤)	Intermediates	Sensitive (≥)
Kanamycin (30 µg)	13	14-17	18
Erythromycin (15 µg)	13	14-22	23
Cinoxacin (100 µg)	15	16-20	21
Cefazolin (30 µg)	14	15-22	23

Hemolytic Test

The hemolytic test was performed on blood agar containing 5% sheep blood. The 24-h LAB cultures were streaked on the blood agar and incubated at 37°C for 24 h [21]. The observed of clear zones indicated hemolytic activity. The isolates with no hemolytic activity were selected for the next assay.

Acidification Activity Test

The acidification activity test was carried out using skim milk media supplemented with 0.3% yeast extract and 0.2% glucose [22]. The 3 mL of selected LAB cultures were inoculated into 27 mL of skim milk and then incubated at 30°C for 24 h. The skim milk without inoculum was used as a control. The sampling was conducted at 0, 2, 4, 6, and 24 h of incubation to evaluate pH change (ΔpH) and calculated using Formula 3. The cultures were considered to have rapid, moderate, or slow acidification rate when ΔpH=0.4 and were achieved after 3, 3-5, or >5 h, respectively.

$$\Delta\mathrm{pH} = \text{initial pH} - \text{final pH} \ldots\ldots\ldots (3)$$

Molecular Identification

The selected isolate was identified using a molecular technique based on 16s rDNA sequences. The total DNA was extracted using the Zymo-Spin™ Lysis Kit following the manufacturer's instructions. The PCR program was run at 95°C for 5 min for initial denaturation, followed by 35 cycles of 95°C for 30 seconds of denaturation, 52°C for 45 seconds of annealing, 72°C for 90 seconds of extension, and the final extension at 72°C for 5 min. The extracted DNA was amplified using 16S rDNA universal primers of 27f (5'-AGA GTT TGA TCC TGG CTC AG-3') and 1492r (5'-GGT TAC CTT CTG ACT ACT T-3'). The amplicon was confirmed using gel agarose electrophoresis (1.5%) and then sequenced at 1st BASE, Malaysia. The sequences were analyzed for constructing a phylogenetic tree using MEGA 6 software with the Neighbor-Joining algorithm and the Tamura-Nei model with a bootstrap of 1000 [17].

Data Analysis

Data of proteolytic activity, lipolytic activity, antibacterial activity, antibiotic sensitivity, and acidification were analyzed by ANOVA with IBM SPSS v.25 software with a 95% confidence level. The data were considered significant when p ≤ 0.05. In addition, the Tukey test was carried out to determine the difference among isolates. Each test was carried out in triplicates.

RESULT AND DISCUSSION

The nutritional contents of buffalo milk play an important role in determining the quality of the milk as well as supporting the growth of indigenous microbes. The proximate analysis of Sumbawa buffalo milk samples (in the average of two samples) revealed that protein content of 5.52%, a fat content of 11.13%, the water content of 77.48%, ash of 0.86%, carbohydrates of 5.01%, and pH of 6.82 (Table 2). The highest protein content in sample S was 6.03%, and the highest fat content in sample T was 12.82%. The buffalo milk samples from North Lintau Buo District, Tanah Datar Regency, West Sumatra, has protein content of 5.23–6.49%, fat content of 7.84–12.44%, the water content of 76.27–80.61%, and pH 5.6–6.0 [23]. It is not much different from the proximate content in Sumbawa buffalo milk, but the pH of Sumbawa buffalo milk was closer to a neutral pH.

Table 2. Proximate content in Sumbawa buffalo milk samples

Parameters	Proximate Content		
	Buffalo Milk Sample S	Buffalo Milk Sample T	Average
Protein (%)	6.03	5.01	5.52
Fat (%)	9.44	12.82	11.13
Water (%)	78.63	76.32	77.48
Ash (%)	0.88	0.84	0.86
Carbohydrate (%)	5.02	5.01	5.01
pH	6.83	6.81	6.82

In general, buffalo milk has protein content of 3.6%, fat content of 7.4%, and lactose content of 5.5% [24]. Therefore, the protein and fat content of the Sumbawa buffalo milk samples were higher than of buffalo from other regions. The Sumbawa buffalo milk has the potential to be used as raw material for developing milk products with a high nutrition. The buffalo found on Sumbawa Island is a swamp or mud buffalo. Swamp buffaloes produce less milk than River buffaloes but have more nutritional qualities, including protein, lean, dry weight, and higher fat than River buffalo [25]. However, further research is still warranted to collect more diverse samples to ensure this result.

Lactic acid bacteria are commonly found as indigenous bacteria in fresh milk, including buffalo milk. It is supported by the result of this study that the total LAB was $4.7x10^5$ CFU.mL^{-1}. This cell density was lower than that of fresh buffalo milk from North Lintau Buo District, Tanah Datar Regency, West Sumatra, which the total LAB reported by $2x10^7$–$1.2x10^8$ CFU.mL^{-1} [23]. It is probably due to the pH difference. The pH of fresh buffalo milk from Lintau Buo Utara District, Tanah Datar Regency, West Sumatra, is lower (5.6–6.0) than the pH in Sumbawa buffalo milk (6.82).

Based on phenotypic characteristics, a total of 21 LAB isolates were obtained. The entire LAB isolates showed Gram positive and catalase negative. The proteolytic activity test was determined for the initial screening stage of LAB as a starter culture. Based on the proteolytic activity, 17 isolates were categorized as high proteolytic activity, including SA8 (5.97 mm), SA1 (5.70 mm), TA6 (5.68 mm), SA9 (5.63 mm), TA12 (5.57 mm), SA7 (5.33 mm), TA5 (5.27 mm), TA8 (5.08 mm), TA11 (4.90 mm), SA2 (4.73 mm), SA4 (4.67 mm), TA9 (4.53 mm), TA1 (4.47 mm), SA3 (4.33 mm), TA2 (4.27 mm), SA6 (4.23 mm), TA4 (4.17 mm) (Fig. 1). Of these isolates, ten isolates exhibiting proteolytic activity of more than 4.70 mm were selected for the next screening stage. In this proteolytic activity, the protein (casein) in milk is hydrolyzed and used as a nutrient. Proteolytic activity plays an important role during the fermentation process. The decomposition rate of polypeptides into peptides and amino acids determines the acceleration of the fermentation process.

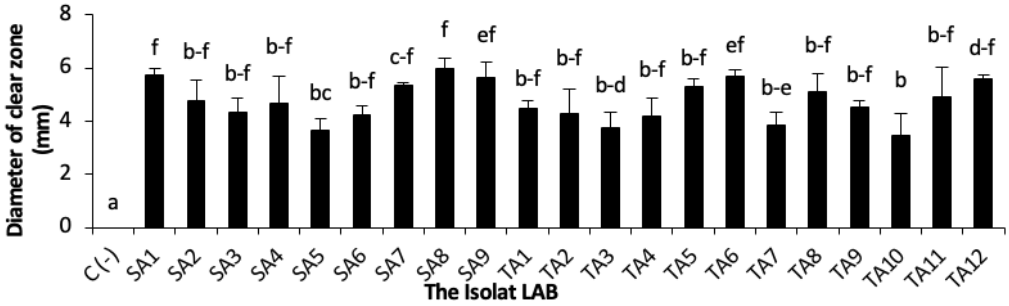

Figure 1. Proteolytic activity of LAB isolates isolated from Sumbawa buffalo milk. The different notation shows a significant difference among isolates ($p<0.05$). **Description:** C (-) = negative control. SA = sample form Srangin Village, 1-9 = isolate number, TA = sample from Tanakakan Village, 1-12 = isolate number.

The LAB proteolytic system plays a key role in the fermentation process, allowing bacteria to grow and adapt inside the milk, thus ensuring a successful fermentation process [27]. Besides supporting bacterial growth, the proteolytic activity of LAB is essential for contributing to the development of organoleptic properties of fermented milk products, especially flavor quality [28]. In fermentation products, the proteolytic bacteria can increase the number of peptides and free amino acids, which affects its flavor. From the perspective of the fermentation industry, the proteolytic properties and the ability of lactic acid production from sugar are the main criteria for determining the starter culture [29]. The selection of starter cultures based on enzyme production and proteolytic activity is essential, in addition to other characteristics such as acidification properties and resistance to high NaCl concentrations [30].

The lipolytic activity was performed to obtain LAB with low activity in utilizing lipids as a carbon source to avoid the rancid flavor. Of 10 selected LAB isolates, seven LAB isolates (TA6, TA11, SA8, SA9, TA5, SA2, and TA12) were selected with low lipolytic activity (Fig. 2).

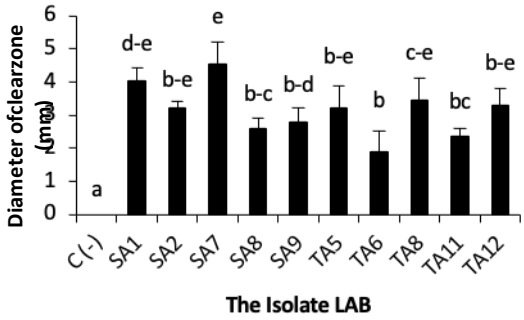

Figure 2. Lipolytic activity of LAB isolates isolated from Sumbawa buffalo milk. The different notation shows a significant difference among isolates ($p<0.05$). **Description:** C (-) = negative control. SA = sample form Srangin Village, TA = sample from Tanakakan Village.

Lipase from LAB contributed to the development of flavors in food [31]. In the case of fermented milk products, except cheese, the rancid odor is categorized as an undesirable flavor. The impact of lipolytic activity can result in the appearance of rancid odors caused by short fat chain reactions such as butyrate [32]. Therefore, high lipolytic activity is desirable for starter culture in cheese production. However, this study was designed to explore the starter culture for fermenting buffalo milk to produce non-cheese products, such as yoghurt and other beverage fermented products. Therefore, the LAB isolates with low lipolytic activity were selected. The seven LAB were confirmed qualitatively in producing exopolysaccharides as indicated by the appearance of ropy or mucoid colonies. The colonies still resembled sticky threads when pulled. However, it is necessary to conduct quantitative analysis to ensure the production of EPS and its concentrations.

Exopolysaccharide-producing bacteria show a ropy or mucoid (slimy) colony [19]. In addition, some LAB produces extracellular capsules or polysaccharides with technological properties and desired biological activity. The polysaccharides produced by LAB are called EPS and can be used to modify rheological properties (viscosity) and play a role in emulsification and flocculation [33]. EPS are produced extracellularly by microbes, and it is connected to the cell in the form of a capsule or mucus of the outside the bacterial cell wall or cell surface. The production of EPS can be easily detected in response to sucrose contained in the media.

However, detection of EPS production cannot be merely dependent on qualitative tests. The LAB isolated from Indonesian traditional foods obtained 108 isolates, and only 60% of isolates produced EPS [34]. Furthermore, from six LAB isolates from Sumbawa fermented mare's milk products, there was only one isolate (BC7 isolate) capable of producing EPS, as indicated by the texture of the ropy colony. The other isolates were classified as soft-type colonies. However, the soft-type colony which did not form sticky threads produced a high EPS concentration which was detected quantitatively [17].

The antibacterial activity test showed the three LAB isolates were able to inhibit the indicator bacteria (*B. cereus*, *E. coli*, and *S.* Typhi), namely SA8, SA9, and TA6 (Fig. 3). Interestingly, seven LAB isolates were able to inhibit the growth of *S.* Typhi, but none can inhibit *S. aureus*. The SA8 isolates exhibited a potential antibacterial activity as it showed the highest inhibitory activity against *E. coli* (1.80 mm) and *S.* Typhi (2.60 mm) but moderately against *B. cereus* (2.27 mm).

Lactic acid bacteria used as starter cultures can extend the products due to the presence of natural antibacterial compounds in the form of organic acids as primary metabolite products, such as lactic, acetic, and formic acids. It leads to a decrease in pH, consequently inhibiting the growth of foodborne pathogens [35]. However, other antibacterial compounds are also produced, such as ethanol, H_2O_2, diacetyl,

reuterin, and bacteriocins. All such antibacterial compounds can resist the growth of pathogenic bacteria in food and control most undesirable organisms [36]. The antibacterial activity shown in this study was the action of non-acid compounds as the neutralized CFS was applied. Therefore, it is suggested to conduct more studies to confirm whether the bacteriocin is responsible for this inhibitory activity.

Some metabolites, such as antibacterial peptides, can play a role in LAB's performance and metabolism, affecting the safety of fermented products. LAB that are often used as starter cultures are *Lactobacillus* spp., *Lactococcus* spp., *Enterococcus* spp., or *Pediococcus* spp. showed a low level of virulence factor, including antibiotic resistance and biogenic amine-related genes, and can be considered safe [30]. Bacteriocins as bactericidal peptides has modes of action, such as pore formation, DNA cell degradation, and inhibition of peptidoglycan synthesis. Bacteriocins can be absorbed by teichoic acid and lipoteichoic acid. These acid compounds are found on the surface of Gram-positive bacteria. The activity of bacteriocins is categorized as a narrow spectrum against bacteria that are still closely related to their producing bacteria [37].

Staphylococcus aureus was the only indicator bacteria that cannot be inhibited. Adha [38] reported that bacteriocin produced by *Lactobacillus plantarum* isolated from curd was not able to inhibit *S. aureus. Staphylococcus aureus* can form layers in the extracellular matrices (biofilm), which might increase tolerance to antibiotics, disinfectants, and resistance to other immunocompetent cells [38].

Based on the antibacterial activity test, three isolates were the potential antibacterial producer (SA8, SA9, and TA6) because they can inhibit three indicator bacteria, and the SA8 isolate showed the most potential antibacterial producer. The selected isolates showed a sensitivity to the antibiotic cefazolin and resistance to kanamycin (Fig. 4). Although the TA6 isolate showed the presence of a clear zone (5.43 mm), based on James's interpretation [20], the zone formed is still categorized as resistant to kanamycin. The SA2 and SA9 isolates were sensitive to erythromycin and cefazolin. But they were resistant to kanamycin and cinoxacin. The TA5 isolate were sensitive to the erythromycin and cefazolin but intermediates to cinoxacin.

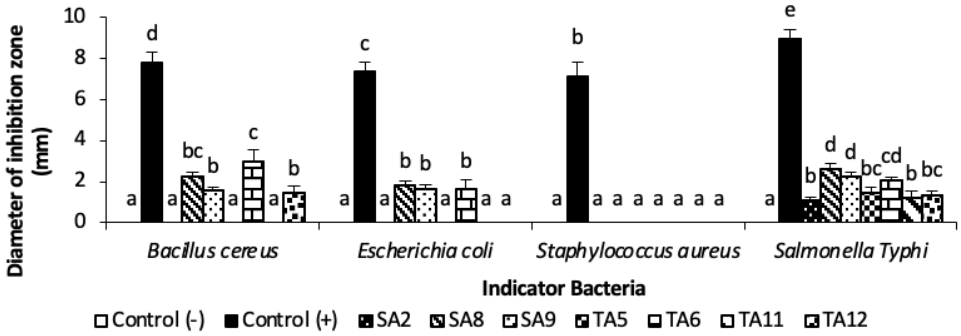

Figure 3. Antibacterial Activity by LAB isolates isolated from Sumbawa Buffalo milk. The notation on the graph shows the real difference of Tukey at p<0.05. **Description:** C (-) = negative control, C (+) = positive control. SA = sample form Srangin Village, TA = sample from Tanakakan Village

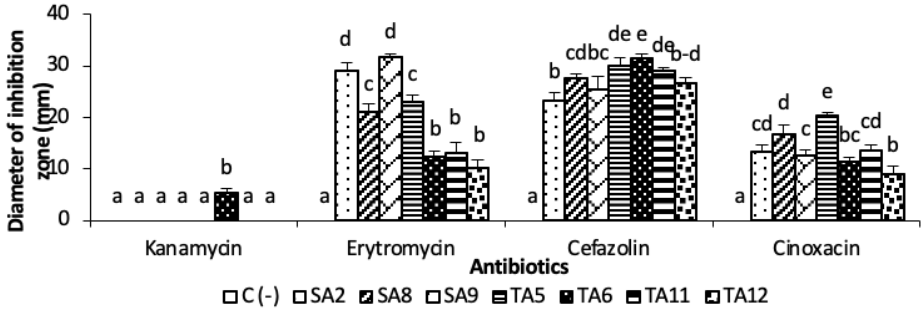

Figure 4. Antibiotic sensitivity of LAB isolates isolated from Sumbawa buffalo milk. The different notation shows a significant difference among isolates (p<0.05). **Description:** C (-) = negative control. SA = sample form Srangin Village, TA = sample from Tanakakan Village.

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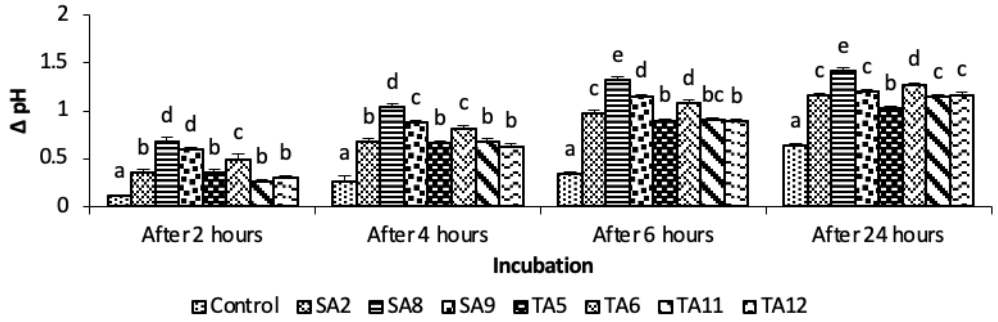

Figure 5. Acidification activity of LAB isolates isolated from Sumbawa Buffalo milk. The different notation shows a significant difference among isolates ($p<0.05$). **Description:** Control = without inoculum. SA = sample form Srangin Village, TA = sample from Tanakakan Village.

The resistance to kanamycin has also been reported before. Four out of six LAB isolates from Sumbawa fermented mare's milk products were resistant to kanamycin [17]. Microbial resistance to antibiotics is caused by the excessive use of antibiotics and causes selective pressure on the proliferation of microorganisms [39].

To confirm the pathogenicity potency, the seven LAB isolates, which have been selected from a range of starter culture criteria before, showed that these LAB isolates did not exhibit lysis activity towards red blood cells contained in the blood agar media. These isolates were categorized as gamma haemolysis. It can be concluded that the selected LAB isolates were safe for use in food production.

The acidification test was the final screening for the starter culture used in this study. The isolates of SA8, SA9, and TA6 were categorized as having very rapid acidification rates with a pH change above 0.4 achieved after 2 h of incubation (Fig. 5). Meanwhile, the other isolates (SA2, TA5, TA6, TA11, and TA12) had moderate acidification activity with a pH change of 0.4 achieved after 4 h of incubation. The isolates of SA8 and SA9 were the fastest in lowering the pH of milk after incubating for 2 h, with pH changes of 0.68 and 0.59, respectively. Meanwhile, the TA11 isolate was the lowest acidification activity, with a pH change being 0.26. The SA8 isolate was the most rapid in lowering the pH of milk at every sampling.

Acidification activity as a key mechanism of fermentation showed a significant influence on the product's sensory profile and the LAB's strain stability. The initial stage of metabolism in fermentation using mixed microorganisms occurs in a very rapid acidification process. It is due to an exchange of several metabolites, indicating a strong acid production capacity. As fermentation progresses, an increase in acid concentration might inhibit the growth of LAB, thereby affecting acid production [40]. Acidification and rapid growth of the LAB starter culture during food fermentation minimize the risk of spoilage and failure of the fermented products. In addition, starter culture plays a major role in the change of taste. Recent studies on different microbial species have shown that high growth rates were influenced by the degree of expression of metabolic enzymes and stress proteins. In starter cultures, such exchanges will affect the formation of sensory profiles and bacterial survival [41]. Based on all screening steps conducted, the SA8 isolate was selected as a potential candidate for a starter culture. Furthermore, the phylogenetic analysis successfully identified this isolate as *Enterococcus lactis* with a similarity level of 99.99% toward *E. lactis* BT159 (Fig. 6).

Genus *Enterococcus* was rarely found in buffalo milk originating from Indonesia. *Enterococcus faecalis* was identified in the milk of Belang Toraja buffalo [21]. On the contrary, Genus *Lactobacillus* was dominantly reported. *Lactobacillus plantarum*, *L. brevis*, *L. pentosus*, and *L. Lactis* were found in buffalo milk from North Sumatra [9]. *Lactobacillus brevis*, *L. paracasei*, *L. pentosus*, *L. plantarum*, and *L. Lactis* were detected in buffalo milk from Pampangan, South Sumatra [42]. Moreover, *L. fermentum* strain NRIC 0129 was reported in buffalo milk from LintauBuo Utara District, Tanah Datar Regency [23]. *Enterococcus* is the main culture found in cheese products, acting as a natural starter during fermentation. It plays an important role in cheese ripening by giving the cheese its distinctive taste and taste [43]. *Enterococcus* is a hardy species and can adapt well to harsh environmental conditions [44].

Enterococcus lactis strains were susceptible to

ampicillin, gentamicin, penicillin, vancomycin, clindamycin, sulfamethoxazole, and chloramphenicol. But it was resistant to erythromycin and tetracycline [45]. Some strains of *E. lactis* were considered potential probiotic strains because they lack a specific virulence and antibiotic resistance gene [46]. *Enterococcus lactis* produces enterocins A, B, and P and exhibits antagonistic activity against pathogenic bacteria and fungi [47].

The SA8 isolate was genetically closely related to *Enterococcus lactis* strain BT159. The strain BT159 was first isolated from Bitto cheese made from cow's and 10% goat's milk. According to Morandi *et al.* [43], this strain was characterized as Gram-positive, facultatively anaerobic, non-motile, non-spore-forming, catalase-negative, coccus-shaped, and arranged in pairs or short chains. The characteristics of the colonies on MRS agar were whitish, smooth, and circular, with a thorough border texture. This strain was able to hydrolyze gelatin and tributyrin, as well as produce gas from glucose. All *Enterococcus lactis* strains can grow on NaCl with concentrations of 2, 4, and 6.5% and withstand temperatures of 10 and 45 °C. They also can grow in litmus milk and cause acidification of cheese at 24-hour incubation [43].

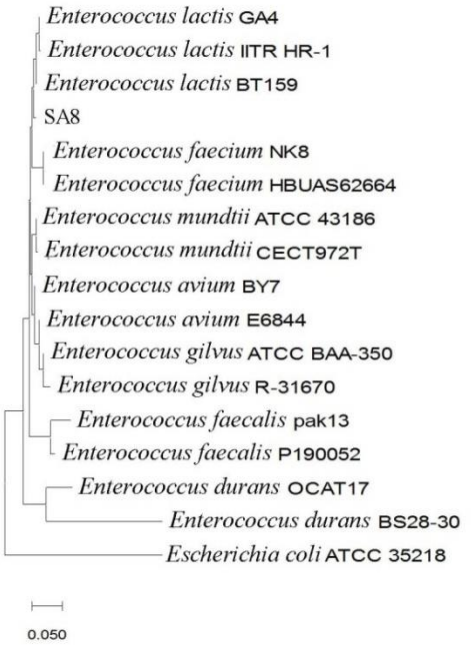

Figure 6. Phylogenetic tree showing the relationship between SA8 isolates and reference bacterial strains based on analysis of 16S rDNA sequences.

CONCLUSION

A total of 21 LAB isolates were obtained from Sumbawa buffalo milk with a total LAB of $4.7x10^5$ $CFU.mL^{-1}$. All isolates were Gram-positive, catalase-negative, and cocci-shaped. The SA8 isolate was selected as the most potential candidate for a starter culture. This isolate has met the starter culture criteria used such as the best ability in proteolytic activity, the lowest lipolytic activity, producing EPS, the best inhibitory activity against *Bacillus cereus*, *Escherichia coli*, and *Salmonella* Typhi, and sensitivity to cefazolin, intermediate to erythromycin and cinoxacin, no hemolytic activity, as well as the fastest acidification rate. The isolate SA8 was identified as *Enterococcus lactis* with a similarity level of 99.99%.

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