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Research Article
Isolation and Identification of Indigenous Lactic Acid Bacteria by Sequencing the 16S rRNA from Dangke, A Traditional Cheese from Enrekang, South Sulawesi

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Abstract

Background and Objective: Dangke is a traditional cheese from South Sulawesi that has been developed by the people of the Enrekang district throughout history. The microbiota of this cheese consists of a wide variety of bacterial species. The majority of which belongs to Lactic Acid Bacteria (LAB) genera. The indigenous LAB of dangke could be a potential source of starter cultures and probiotics. The aim of this study was to isolate the LAB from dangke and identify them by 16S rRNA sequencing. **Methodology:** The dangke from Enrekang, South Sulawesi were collected. The LAB were identified by morphology (Gram staining and cell form), physiology (growth and viability in 6.5% NaCl and temperatures of 15, 37 and 45°C), biochemistry (catalase-negative test and CO_2 production) and survival at low pH (2, 3, 4 and 7.2) and in bile salts (0.3%). **Results:** The results showed that 30 isolates were identified as LAB with Gram-positive, catalase-negative and rod-shaped characteristics. Ten LAB isolates from dangke had highest tolerance to low pH and bile salts. The isolates that were resistant to low pH and bile salts were A123K, A113L, A323L, B111K, B212K, B221L, B312K, B323K, C113L and C222L. The 16S rRNA gene could be amplified by Polymerase Chain Reaction (PCR) from 5 isolates (A323L, B111K, B323K, C113L and C222L) to obtain a single band on a 1% agarose gel. **Conclusion:** Identification by 16S rRNA gene sequencing showed all isolates were identified as *Lactobacillus fermentum* with a similarity index of approximately 99-100%.

Key words: Dangke, isolation, identification, lactic acid bacteria, 16S rRNA

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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

Dangke has been produced by the Enrekang people since 1905. Dangke is made from cow or buffalo milk. It is processed by pasteurization and coagulated with the papain enzyme to separate the curd and whey. The curd is then placed in a special mold made from a coconut shell and is pressed until the whey is separated[1]. Dangke contains a wide variety of microbiota, particularly Lactic Acid Bacteria (LAB) genera. The LAB are generally used for starter cultures that produce lactic acid as a main product and other metabolites, such as aroma compounds, which contributed to the flavor and product texture. Moreover, LAB could increase the solubility of carbohydrates and the sweetness of the final product[2-3].

The LAB can also be developed into probiotics for functional food. Probiotics are living micro-organisms that, if by humans or animals in a sufficient amount will confer health benefits to the host by improving the intestinal microbiota balance[4]. Many reports observed the potential of LAB as probiotics for human. The LAB could produce antimicrobial compounds[5-7], reduce the serum cholesterol levels[8,9], prevent "Lactose intolerance", exert antihypertensive effects[10], stabilize the intestinal microflora[11], exert antioxidant effects, enhance the systemic immune response[12] and exhibit anticancer and antitumor activity[13]. Probiotics can be applied to food products such as fermented sausage[14], milk products[12,15,16], soy milk[17] and fermented whey[18].

The isolation of indigenous Lactic Acid Bacteria (LAB) from dairy and dairy products has frequently been reported, including LAB from raw cow milk[19-21], buffalo milk[22], goat milk[23-25], camel milk[26-28], Sumbawa mare milk[29], breast milk[30,31] and traditional cheese and dairy products[8,32-36]. In Indonesia, Sunaryanto and Marwoto[37] reported that the isolation of LAB from a traditional dairy product called "Dadih". However, studies on the properties of LAB isolated from dangke have not been reported. The aim of this study was to isolate the LAB from dangke and identify them by 16S rRNA sequencing. To our knowledge, this is the first report on the identification of LAB from dangke.

MATERIALS AND METHODS

Sampling: A total of 9 samples of dangke were collected from different regions of the dangke home industry in Enrekang district, South Sulawesi. The samples were immediately collected by aseptic technique and then transported to the laboratory in a cooling box (4°C) to minimize contamination. The samples were analyzed to determine the LAB content upon the arrival.

LAB isolation: Twenty-five milligram samples of dangke were homogenized with 225 mL of a 0.85% (w/v) sterile, physiological NaCl solution (10^{-1}). Serial dilutions were made for each sample and 1 mL of the appropriate dilution (10^{-4}, 10^{-5} and 10^{-6}) was spread on MRS agar plates (Merck KGaA, Darmstadt, Germany) and then incubated at 37°C for 48 h. After incubation, colonies with distinct morphologies (such as color, shape and size) were randomly selected, purified by streaking them on the MRS agar and then incubated at 30°C for 48 h. The colonies were continuously streaked to obtain a pure colony[38].

Characterization of the LAB isolates: All isolates were characterized according to their morphology by Gram staining and cell formation[8], their physiology by growth and viability at 6.5% NaCl and temperatures of 15, 37 and 45°C[38], their biochemistry by the catalase-negative test and CO_2 production[39] and their survival at low pH (2, 3, 4 and 7.2)[29] and in 0.3% (b/v) bile salts[25,40].

Analysis of the 16S rRNA gene sequences

DNA extraction: The total genomic DNA of each isolate was extracted from a 5 mL culture of each pure colony grown in MRS broth (Merck KGaA, Darmstadt, Germany) overnight at 37°C. The DNA was extracted as described by Sambrook and Russell[41] with some modifications. The cultures were centrifuged at 10.000×g for 1 min at 37°C and the supernatant was discarded. Then, 200 µL of solution I (25 mM Tris-HCl buffer, pH 8.0, 10 mM EDTA and 50 mM glucose) were added to the cells, which were resuspended by pipetting and incubated for 5 min at room temperature. Next, 400 µL of solution II (1.2 N NaOH and 1% SDS) were added and the solution was mixed gently and incubated on ice for 5 min. Subsequently, 300 µL of solution III (60 mL of 5 M potassium acetate, 11.5 mL of acetate acid and 28.5 mL of dH_2O) were added, vortex mixed and then incubated on ice for 5 min. Ten microliters of chloroform were added to the mixtures, vortex mixed and centrifuged at 10.000×g for 10 min at 4°C. The supernatant was transferred to a new tube (1.5 mL) and 1 µL of 1 mg mL^{-1} RNase A was added, mixed gently and incubated at room temperature for 15 min. Then, 500 µL of 2-propanol was added, mixed gently and centrifuged at 10.000×g for 10 min at 4°C. The supernatant was then discarded and 500 µL of ethanol (70%) was added, mixed gently and centrifuged for 1 min. Then, the supernatant was discarded to collect the purified DNA, which was then dried. The purified DNA was resuspended with 100 µL of Tris EDTA (TE) buffer for further applications.

16S rRNA gene sequencing: The genomic DNA was use as a template for PCR amplification of a segment of its 16S rRNA gene. The two universal primers used in this study were the same as those described in a previous report[38], it namely 9 F (5'-GAGTTTGATCCTGGCTCAG-3') and 1541 R (5'-AGGAGGTGATCCAGCC-3'). The PCR amplification was performed to obtain a single band representing the amplified 16S rRNA gene product. The PCR products were examined by 1% agarose electrophoresis, photographed using a UV Transilluminator and printed on Polaroid film[38].

Data analysis and species identification: The nucleotide sequences of the 16S rRNA genes from all LAB isolates were analyzed and identified using the GenBank data library and BLAST program on the NCBI website http://www.ncbi.nlm.nih.gov/[18]. Multiple sequence alignments were analyzed using BioEdit software to construct a phylogenetic tree and to compare similarities among the sequences by the neighbor-joining method using MEGA 7 software[42].

RESULTS

Isolation and Identification of LAB: Thirty pure isolates were obtained from dangke and were identified as LAB based on their morphology, physiology and biochemical tests. All isolates grew on MRS agar under aerobic conditions and were Gram-positive and negative for catalase activity, indicating that those isolates belonged to the genus *Lactobacilli*. The morphological, physiological and biochemical profiles of isolated LAB were shown in Table 1.

Characterization of the isolates: The morphological characteristic of the LAB isolates were identified by Gram staining, which showed that all isolates were Gram-positive bacteria. The analysis of the shapes of the LAB showed that 28 isolates exhibited the rod form and 2 isolates displayed the oval form (Table 1). The test of the biochemical characteristics showed that all isolates were catalase-negative and 70% of the LAB could produce gas from glucose, indicating that 70% of the LAB isolates were heterofermentative *Lactobacilli* and

Table 1: Morphological, physiological and biochemical profiles of LAB

				Growth (°C)				
Isolate codes	Shape	Gram stain	Catalase	10	37	45	Growth in 6.5% NaCl	Gas from glucose
A112K	Rod	+	-	+	+++	++	+	-
A113L	Rod	+	-	+	+++	+++	+	+
A123K	Rod	+	-	-	+++	+++	+	+
A123L	Rod	+	-	+	+++	++	+	-
A212K	Rod	+	-	+	+++	++	+	-
A212L	Rod	+	-	-	+++	+++	+	+
A312K	Rod	+	-	+	+++	+++	+	+
A321K	Oval	+	-	-	+++	++	+	-
A321L	Rod	+	-	+	+++	++	+	-
A323L	Rod	+	-	+	+++	+++	++	+
B111K	Rod	+	-	-	+++	+++	++	+
B122K	Rod	+	-	+	+++	++	++	+
B212K	Rod	+	-	+	+++	++	++	+
B211K	Rod	+	-	+	+++	++	++	+
B221K	Rod	+	-	+	+++	++	++	+
B221L	Rod	+	-	+	+++	+++	++	+
B322L	Rod	+	-	+	+++	+++	+	+
B312K	Rod	+	-	+	+++	+++	++	+
B321K	Rod	+	-	+	+++	+++	+	+
B323K	Rod	+	-	+	+++	+++	++	-
C111K	Rod	+	-	+	+++	++	++	+
C112K	Oval	+	-	+	+++	++	++	+
C113L	Rod	+	-	+	+++	++	+++	-
C121L	Rod	+	-	+	+++	++	+++	-
C123K	Rod	+	-	+	+++	++	++	+
C211K	Rod	+	-	+	+++	+	++	+
C213K	Rod	+	-	+	+++	+++	++	+
C221L	Rod	+	-	+	+++	++	++	+
C222L	Rod	+	-	+	+++	+	++	+
C312L	Rod	+	-	+	+++	++	++	-

LAB: Lactic acid bacteria, A: Anggeraja, B: Enrekang, C: Cendana, +: Weak growth, ++: Medium growth, +++: Strong growth, -: No growth

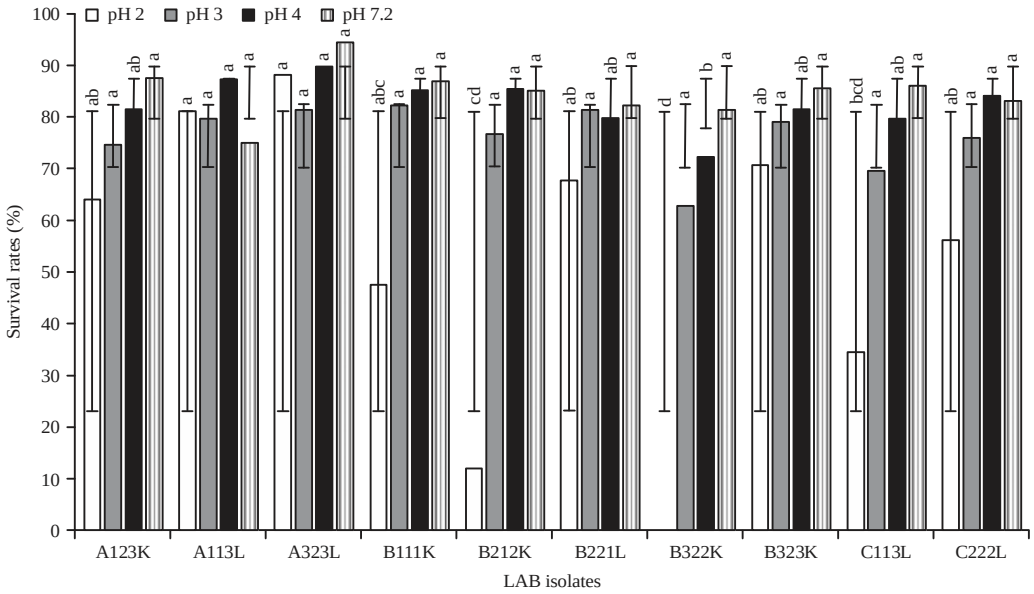

Fig. 1: Survival rates of the LAB isolates at different pH values

LAB: Lactic acid bacteria

30% were homofermentative *Lactobacilli*. The lactic acid bacteria could be divided into homofermentative and heterofermentative bacteria based on their ability to ferment glucose. The heterofermentative LAB could produce gas as an end product of fermentation.

The test of the physiological characteristics showed that all isolates could grow well in 6.5% NaCl, 36.67% of the isolates showed weak growth, 56.67% showed medium growth and 6.67% showed strong growth. According to the temperature test, all isolates could grow well at high temperature (45°C), except for the C211K and C222L isolates, which showed weak growth. Furthermore, 26 out of 30 (86.67%) isolates could grow at low temperature (10°C). One factor that affected the bacterial growth was temperature. Bacteria can be categorized as psychrophilic, mesophilic and thermophilic, according to their range of temperature growth. Some species of LAB may grow well at high temperatures[43]. Thermophilic *Lactobacilli* and *cocci* groups can grow at 45°C, but can not grow at 10°C or in 6.5% NaCl (w/v). Mesophilic groups are able to grow at 10°C, but cannot grow at 45°C or in 6.5% NaCl (w/v). However, *Enterococci* groups are able to grow at 45 and 10°C and in 6.5% NaCl[44].

The BAL isolates were grown in acidic pH media (2, 3 and 4) and normal pH media (7.2) to explore the viability of LAB under acidic conditions. The results showed that viability of LAB at acidic pH was significantly different ($p<0.05$) at pH 2, 4 and 7.2, but not significantly different ($p<0.05$) at pH 3. The survival of the LAB isolates at pH 2, 3, 4 and 7.2 were shown in Fig. 1. A total of 10 LAB isolates could survive in the acidic pH conditions, but their survival abilities were different and only 3 isolates (A113L, A323L and B323K) had good survival rates (>70%) at pH 2. The survival rates of all LAB isolates at pH 4 were better than those at pH 3 and 2, which were above 70%, with a range of 81.47-91.12%. The survival rate of the A323L isolate (88.63%) at pH 2 was even better than some isolates at pH 3, 4 and 7.2. All isolates were able to survive at pH 2 and 3 and showed better survival at pH 4 (>68%), some of them showed survival rates close to 100% (the A323L isolate with a survival rate of 97.2%) at pH 7.2. However, the B322K isolate showed a 0% survival rate at pH 2.

Bile salt resistance test of LAB isolates was performed to determine the ability of LAB isolates to resist the bile salts that are normally produced in the intestine and survive and colonize the intestine upon entry[38]. In this study, all LAB isolates could survive well in the 0.3% bile salt solution (Fig. 2). The survival rates showed that all LAB isolates were highly resistant to bile salts (>50%). The results showed that among all isolates, the A232L isolate exhibited the highest survival rate in 0.3% bile salt (83.11%), whereas the C222L isolate exhibited the lowest survival rate of 57.28%.

16S rRNA gene sequences and phylogenetic analysis: The LAB isolated form dangke will be more accurately identified to the species level by 16S rRNA gene sequencing. In this study, the 16S rRNA gene sequences were successfully amplified from 5 isolates by Polymerase Chain Reaction (PCR). The results indicated that the 16S rRNA gene sequences (continuous stretches of approximately 1,500 bp) were

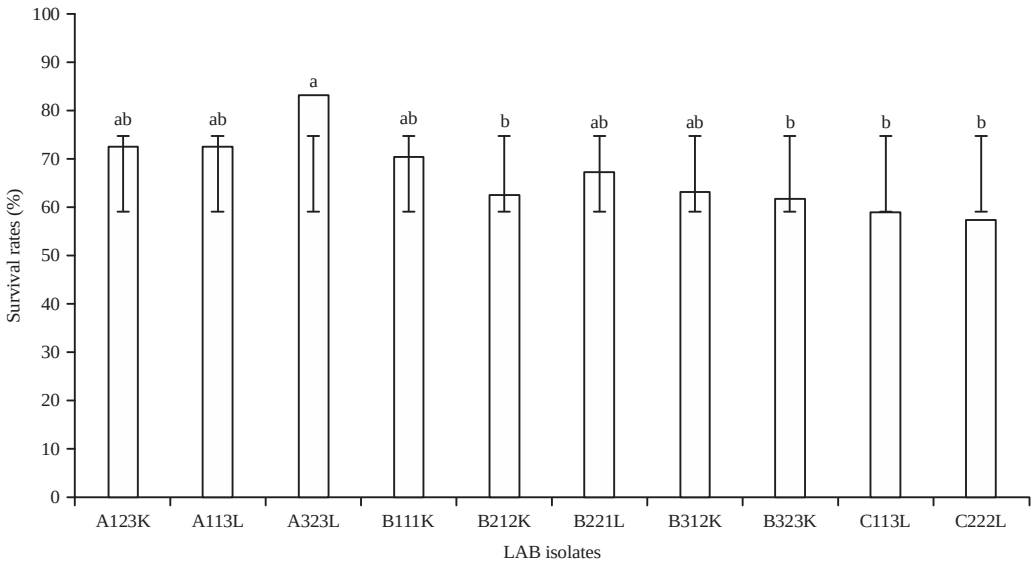

Fig. 2: Survival rates of the LAB isolates in a 0.3% bile salt solution

LAB: Lactic acid bacteria

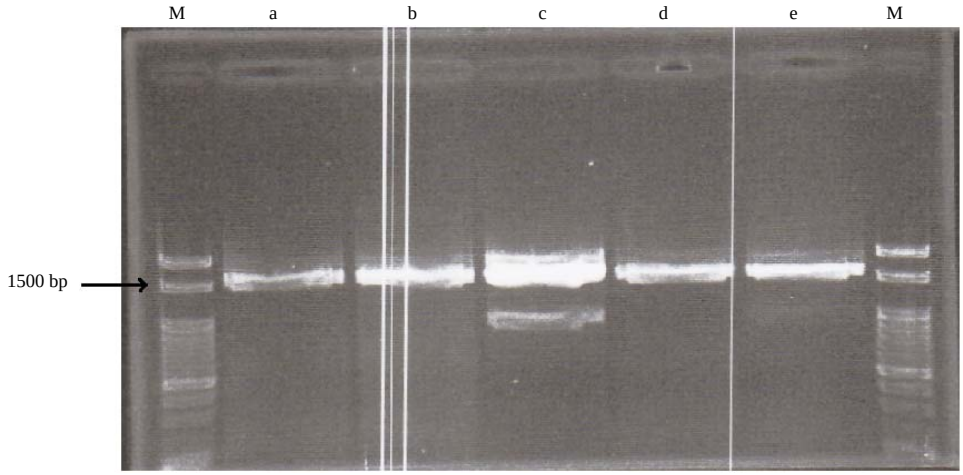

Fig. 3: 16S rRNA was amplified by PCR and separated by 1% agarose gel electrophoresis

M: Marker λEcoT141, a: B323K, b: B111K, c: A323L, d: C222L, e: C113L

successfully amplified with universal primers 9 F and 1541 R (Fig. 3). Moreover, the construction of phylogenetic tree showed that 5 isolates were included in the *Lactobacillus fermentum* group, whose tree is mainly composed of four clusters (Fig. 4).

Two strains, the B323K and C113L isolates were classified in a cluster with *L. fermentum* strain FMAC283 (isolated from a traditional caciocavallo palermitano-type cheese, Italy), *L. fermentum* strain KF7 (isolated from fresh milk, Pakistan), *L. fermentum* strain W5 (isolated from Wara, an African soft cheese) and *L. fermentum* strain LFW2 (isolated from breast milk, Taiwan), where they have a similarity index of 100% based on NCBI BLAST proximity. The A323L isolate was included in a cluster with *L. fermentum* strain 3872 (isolated from the breast milk from a healthy woman, Russia) and *L. fermentum* strain NM985 (isolated from a dairy product, Mongolia), based on an NCBI BLAST proximity of 99.1%. The B111K isolate was included in the cluster with *L. fermentum* strain PD2 and CPO 7.002 that were isolated from traditional dairy products from India (Dosa batter) and Mexico (Jarocho fresh cheese), respectively. They have a 100% proximity based on NCBI BLAST. In the last cluster, the C222L isolate was included in the cluster with *L. fermentum* culture collection IMAU: 80800 from China and had a closed relation of 99.7% according to the NCBI BLAST.

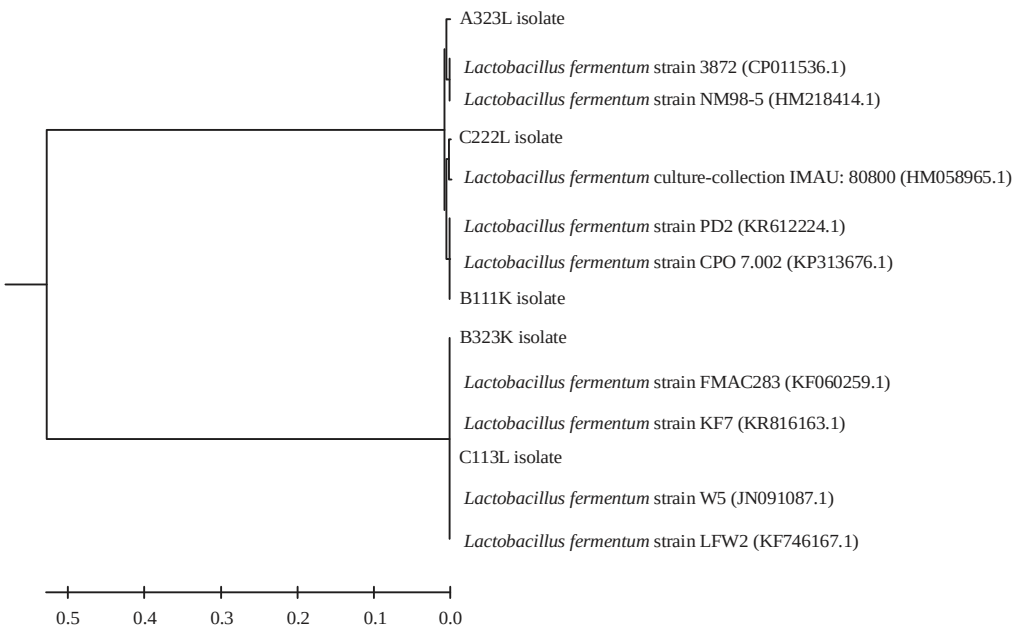

Fig. 4: Phylogenetic tree based on the analysis of the 16S rRNA gene sequences and the closest international isolate showing the phylogenetic placement of the representative strain isolated from dangke

DISCUSSION

The LAB are naturally present as indigenous microflora and are normally regarded as spoilage organisms in raw milk and dairy products[19,45]. They are widely distributed in raw milk and dairy products with potential as probiotics[8,21,46]. The viable counts of LAB among all sample of dangke in this study ranged from 4.03-4.45 log CFU g^{-1}. In similar studies, Liu *et al.*[42] reported that the viable counts of LAB from Tarag (the traditional dairy product form Mongolia) ranged from 4.02-8.88 log CFU mL^{-1}. Moreover, similar counts were reported in commercial LAB products[47] and in other types of milk, such as camel[28] and goat milk[23]. However, it was lower than the viable counts of LAB from dadih (traditional cheese from Sumatera, Indonesia)[37]. It is important to note that the LAB micro-organisms must be viable, active and abundant, with a concentration of at least 6 log CFU g mL^{-1} in the product throughout the specified shelf life to exert a positive effect[42].

Lactobacilli are the LAB that are most frequently used in fermented animal products as a health promoter and are used as probiotics in food, cultured milk and various pharmaceutical preparations[48]. Probiotic bacteria were defined as live micro-organism with health benefits to their host by improving the intestinal microbiota when administered in adequate amounts[4]. Many reports observed the utility of LAB as probiotics for human health[8,28,49-52]. The basic conditions for the use of LAB strains as probiotics minimally include the following: (1) They should be Generally Recognized As Safe (GRAS), (2) They should be tolerant to acids and bile salts and (3) They should be have antagonistic activity against pathogenic bacteria (FAO and WHO)[4], such as those that produce the antimicrobial substances known as bacteriocin, which was used as a natural food preservative[6-7]. In this study, it was isolated the LAB strains from dangke, a traditional cheese from Enrekang, South Sulawesi, identified them as *L. fermentum* through a combination of phenotypic and genotypic tests and investigated their survival rates in both acidic and bile salt solutions.

The *Lactobacillus* genus has been identified as a major species in dairy and dairy products. In this study, it was also found that all the isolates obtained under our experimental conditions were members of the *Lactobacilli* genus. Subsequently, five LAB isolates from dangke were more accurately identified to the species level by 16S rRNA gene sequencing. According to the nucleotide sequence, all isolates were identified as *L. fermentum* (Fig. 4). *Lactobacillus fermentum* has also been isolated from mare milk in Sumbawa by 16S rRNA identification with a sequence length of approximately 440 bp[29]. It was also isolated from traditional cheese[33,53,54], traditional dairy products[55,56] and breast milk[57,58].

The test of resistance to acidic conditions at low pH was accepted as one of the desirable properties used to select potentially probiotic strains of LAB[38]. The candidate probiotic LAB should not only be tolerant to acidic conditions (acidic of the gastric conditions) but also be resistant to intestinal bile salts[46]. The acidic levels of the gastric fluid ranged from pH 1.5-4.5[29] and the bile salt levels in the intestine ranged from 0.15-0.6%[59], depending on food ingestion. In this study, the LAB isolates were tested at pH 2, 3 and 4 (to simulate the gastric conditions) and 7.2 (to simulate the intestinal conditions). The LAB isolated from dangke have different capabilities of surviving acidic conditions. Six isolates had good survival rates (over than 50%) at pH 2 and 4 isolates had survival rates less than 50%. All LAB isolates had survival rates of more than 60% at pH 3, 4 and 7.2. The survival rates of the LAB isolates were then determined after exposure to a 0.3% bile salt solution for 3 h exposure and the survival rates of all isolates were more than 50%. These results indicated that all LAB isolated from dangke can survive well in both the gastric and intestinal conditions. In similar studies, Arief *et al.*[38] reported that survival rates of LAB isolates at pH 2.5 were greater than those at pH 2, which were more than 40%. Moreover, Shi *et al.*[29] reported that more than 50% of *L. fermentum* and *L. rhamnosus* strains survived at pH 2.

CONCLUSION

A total of 30 LAB isolates were successfully isolated from dangke, an Enrekang traditional cheese and 5 isolates were successfully identified by 16S rRNA gene sequencing. The analysis of the 16S rRNA gene sequences showed that all LAB isolates were identified as *Lactobacillus fermentum*. The LAB isolates showed different abilities to survive under gastric conditions. Only 6 LAB isolates could survive well at pH 2 (a survival rate of more than 50%) and the populations increased at pH 3, 4 and 7.2. All LAB isolates could survive well (a survival rate of more than 50%) in a 0.3% bile salt solution. These findings suggest the possibility that LAB isolates from dangke can be used as probiotics. Nevertheless, further studies are needed to identify the other probiotic characteristics of the LAB isolates.

SIGNIFICANCE STATEMENT

This study discover the LAB specifically isolated from dangke and identify them by 16S rRNA sequencing. The identified LAB have been tested and proved to have some probiotic characteristics that can be used as probiotics starter for functional food products. This study will help the researcher to uncover the benefits of indigenous LAB from dangke that many researchers were not able to explore.

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