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RESEARCH ARTICLE
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The Use of Multiplex PCR Reactions to Identification of Lactic Acid Bacteria of Dangke

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Abstract:

Lactic acid bacteria can be isolated from various types of milk including dairy products. Dangke is an Indonesian traditional cheese made by Enrekang community, South Sulawesi. This study aims to identify the lactic acid bacteria by multiplex PCR method which are isolated from dangke obtained from Enrekang Regency. This type of research used descriptive research with laboratory observation methods was performed on 30 cups of dangke are obtained from the markets in 5 sub-districts producing dangke in Enrekang Regency: they are the sub-districts of Cendana, Enrekang, Anggeraja, Alla, and Baraka. The results showed that *Lactobacillus acidophilus* was the most identified (33.33%) compared to *Lactobacillus plantarum* (16.67%). Based on the results of the research that has been carried out, it can be concluded that the lactic acid bacteria have been identified in dangke samples obtained from several sub-districts producing dangke in Enrekang Regency are *Lactobacillus acidophilus* and *Lactobacillus plantarum* species. Thus, we suggest that this laboratory technique is used to confirm the various species of lactic acid bacteria so it can prove more rapid and exactly that dangke is the rich content of lactic acid bacteria as probiotic candidate.

Keywords: Lactic Acid Bacteria; Multiplex PCR; Dangke

Introduction

At the turn of the 20th century the term "lactic acid bacteria" (LAB) was used to refer to "milk-souring organisms".[9] Most probiotic bacteria belong to the group of lactic acid bacteria (LAB) and generally the genus Lactobacilli and Bifidobacteria which play an important role in maintaining the balance of intestinal microflora and stimulating the host immune system.[20] Lactic acid bacteria can be isolated from habitat rich in nutrients such as milk, cheese, meat, beverages and vegetables.[25]

Dangke is a traditional cheese from South Sulawesi Province in Indonesia. Dangke is mostly made from cow's milk but buffalo's milk or their mixture can also be used. A small amount of papain has been used to coagulate casein from whey.[18] LAB which was isolated from buffalo milk dangke from Curio Subdistrict, Enrekang Regency consisted of two species, namely *Lactobacillus plantarum* and *L. fermentum*, both of which have potential as probiotic candidates.[13] Meanwhile, LAB which was successfully isolated from cow's milk dangke from Enrekang Regency were *Lactobacillus acidophilus* and *L. fermentum*.[14]

LAB that has been identified from dangke in previous studies using the culture method and through a series of biochemical tests. However, there are several drawbacks to using this culture method, including time efficiency and identification costs. This is because the LAB population in dangke can be of various species. To answer the shortcomings and limitations of this identification system, a number of studies have recently been reported to develop a faster and more precise system with the help of various molecular biology techniques because this technique is more sensitive and more specific, one of which is by using the Polymerase Chain Reaction (PCR) technique which is quite useful and can be done quickly in public laboratories.[12]

Multiplex PCR in identifying Lactobacillus and Bifidobacterium in several probiotic products can be identify 4 different LAB species and has been verified by DNA sequencing.[22]

Based on the above, this study aims to identify lactic acid bacteria using the multiplex PCR method isolated from dangke from Enrekang Regency, South Sulawesi.

Research Method

Design, Location and Time

The research was conducted in Enrekang Regency, South Sulawesi with 30 cups of dangke samples obtained from markets in 5 (five) sub-districts, namely Cendana, Enrekang, Anggeraja, Alla, and Baraka. Each dangke sample was taken about 0.5 mg to be inserted into a 1.5 ml Eppendorf tube containing L6 solution and then stored at room temperature which would then be extracted for PCR testing.[10] The PCR technique was carried out at the Laboratory of Molecular Biology and Immunology, Department of Microbiology, Faculty of Medicine, Hasanuddin University Makassar and was carried out from May to July 2018. This study used descriptive research with laboratory observation methods.

Materials and Tools

The materials and tools used consisted of dangke, MRSB powder (deMann Rogosa and Sharpe Broth), agarose powder, 70% alcohol, sterile distilled water, cotton, tissue, label, 70% ethanol, acetone, L6 solution (Tris-HCl, GuSCN, EDTA, Triton-X-100), L2 solution (Tris-HCl, GuSCN), silica (SiO2), Tris-EDTA, Tris-HCl buffer, EDTA solution, NaOH solution, 1% SDS, potassium acetate, acetic acid, RNA-ase, Tris EDTA buffer, 16S rRNA gene primer: forward primer All *Lactobacillus* IDL04F (5'-

AGGGTGAAGTCGTAACAAGTAGCC-3') and reverse primer All *Lactobacillus* IDL03R (5'-CCACCTTCCTCCGGTTTGTCA-3'), *L. acidophilus* IDL22R (5'-AACTATCGCTTACGCTACCACTTTGC-3') size 606 bp, *L. plantarum* IDL62R (5'-CTAGTGGTAACAGTTGATTAAAACTGC-3') size 428 bp, ethidium bromide, TBE buffer, micropipette and tip, cooling box , plastic wrap, Eppendorf tube, tweezers, vortex, Falcon tube, aluminum foil, balance scale, stirring spoon, watch glass, beaker, Erlenmeyer glass, petri dish, bunsen burner, ose (round and straight), object glass, test tube, incubator, spray bottle, refrigerator, water bath shaker, PCR workstation/cabinet (Scie-Plus)/Biosafety cabinet, GeneAmpPCR System 9700 (Applied Biosystem), UV transulaminator, Sub Cell GT Electrophoresis System, Profuge Gk-Centrifuge , ice maker (Memmert), Gel Doc XR Model 785, Refrigerated Centrifuge, centrifuge tube, GD column (spin column), MPW-260R centrifuge, PCR machine (DNA thermal cycler).

Sample preparation

Sample identified with culture method into MRSA media (deMann Rogosa and Sharpe Agar) which was added with 1 % $CaCO_3$ and then incubated for 48 hours and molecular method with multiplex PCR. The culture method aims to confirm the presence of LAB which is characterized by growing colonies apart and forming a clear zone on the growth medium, while the molecular method aims to confirm the species of LAB present in the dangke sample.[15,16,23]

DNA extraction, PCR amplification, and Agarose gel electrophoresis

First, the dangke sample that has been put into an eppendorf tube, then DNA extraction is carried out according to the technical instructions carried out by Boom et al.[3] and Bergallo et al.[2] Furthermore, the extracted DNA was amplified by means of a PCR master mixture prepared (for a volume of 40 samples) by inserting the following materials into each Eppendorf Go PCR Beads tube: 200 µl RNA-se free water (5 µl), 40 µl 10× buffer PCR (150 mM Tris-HCl pH 8, 500mM KCl, 0.1% (v/v) Tween 20) (1 µl), 25 mM $MgCl_2$ (0.5 µl), 10 µl dNTP-mix (25 mM per dNTP) (0.25 µl), 10 µl forward primer (20 µM) IDL04F (5'-AGGGTGAAGTCGTAACAAGTAGCC-3') (0.25 µl), 10 µl reverse primer (20 µM) IDL03R (5'-CCACCTTCCTCCGGTTTGTCA-3')(0.25 µl), 10 µl reverse primer (20 µM) IDL22R (5'-AACTATCGCTTACGCTACCACTTTGC-3')(0.25 µl), 10 µl reverse primer (20 µM) IDL62R (5'-CTAGTGGTAACAGTTGATTAAAACTGC-3')(0.25 µl), 10 µl of Taq DNA polymerase (5 U/µl) (0.25 µl) and 2 µl of DNA template to reach a total volume of 10 µl. The tube is then placed in a thermal cycler (Applied Biosystems by Life Technologies) then the machine is turned on to perform the PCR cycle as follows. Initial denaturation occurred at 94°C for 2 minutes, followed by 35 amplification cycles lasting 20 seconds at 94°C , annealing for 40 seconds at 51°C , extension for 30 seconds at 68°C and final extension at 68°C for 7 minutes. The amplification results were analyzed using electrophoresis in agarose gel. Finally, to determine the results of DNA amplification, an electrophoresis process was carried out on the PCR product on 2% agarose gel and the electrophoresis device was run by flowing 150 volts of electricity for 45-60 minutes.

The data processing and analysis technique in this study was the data obtained and then grouped based on the purpose and type of data then analyzed using a 2x2 table. The results of the analysis will be displayed in the form of a table with an explanation.

Results

Sample preparation

Based on Table 1, it is known that the examination of culture using MRSA media added with $CaCO_3$ 1 % was incubated 2 x 24

hours at 37°C from 30 samples there were 28 samples identified as growing colonies of lactic acid bacteria marked by the presence of a clear zone on the medium and forming single colonies and 2 other samples identified did not grow colonies of lactic acid bacteria.

DNA extraction, PCR amplification, and Agarose gel electrophoresis

Based on Table 2 and Figure 1, it is known that of the 30 samples identified molecularly using multiplex PCR, there were 14 samples identified by *Lactobacillus spp*., namely *Lactobacillus acidophilus* and *Lactobacillus plantarum* according to the primer used and the other 16 samples were not identified by the primer used.

Table 1 Identification of lactic acid bacteria by culture method using MRSA media

No	Coloni Codes	Locations and Sources of Coloni					
		A	B	C	D		E
		SS	SS	SS	SK	SS	SS
1	01	+					
2	02	+					
3	03	+					
4	04	+					
5	05	+					
6	06		+				
7	07		+				
8	08		+				
9	09		+				
10	10		+				
11	11			+			
12	12			+			
13	13			+			
14	14			+			
15	15			+			
16	16				+		
17	17				+		
18	18				-		
19	19				+		
20	20				+		
21	21					+	
22	22					+	
23	23					+	
24	24					+	
25	25					+	
26	26						+
27	27						+
28	28						-
29	29						+
30	30						+

Note. (-) : colony did not grow, (+) : colony grew, A : Central Market Enrekang Kec. Enrekang, B : Kabere Market Kec. Cendana, C : Sudu Traditional Market, Kec. Alla, D : Cakke Traditional Market, Kec. Anggeraja, E : Citra Baraka Market, Kec. Baraka, SS: dangke cow's milk, SK: dangke buffalo's milk

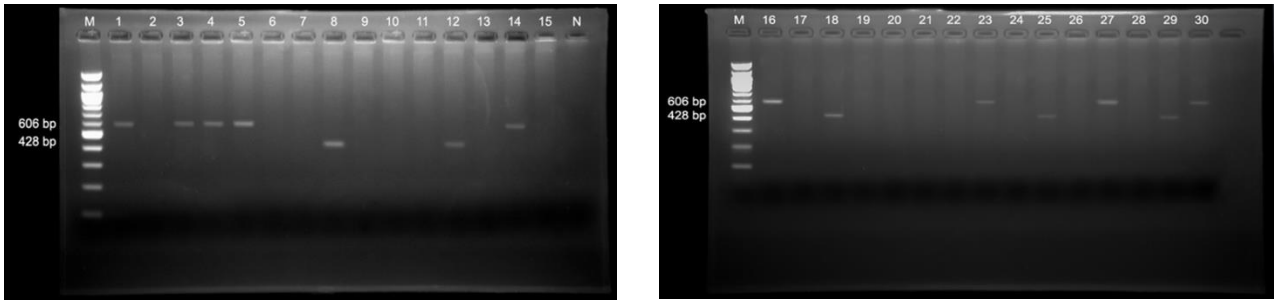
Table 2 Identification of lactic acid bacteria by Multiplex PCR method

Slot	Samples Code	Results		Species
		IDL22R 606 bp	IDL62R 428 bp	
1	M	-	-	-
2	01	+	-	*L.acidophilus*
3	02	-	-	-
4	03	+	-	*L.acidophilus*
5	04	+	-	*L.acidophilus*
6	05	+	-	*L.acidophilus*
7	06	-	-	
8	07	-	-	
9	08	-	+	*L.plantarum*
10	09	-	-	
11	10	-	-	
12	11	-	-	
13	12	-	+	*L.plantarum*
14	13	-	-	
15	14	+	-	*L.acidophilus*
16	15	-	-	
17	16	+	-	*L.acidophilus*
18	17	-	-	
19	18	-	+	*L.plantarum*
20	19	-	-	
21	20	-	-	
22	21	-	-	
23	22	-	-	
24	23	+	-	*L.acidophilus*
25	24	-	-	
26	25	-	+	*L.plantarum*
27	26	-	-	
28	27	+	-	*L.acidophilus*
29	28	-	-	-
30	29	-	+	*L.plantarum*
31	30	+	-	*L.acidophilus*

Note. M : Marker, (-) : colony did not grow or was not detected by primer, (+) : colony grew or detected by primer

Table 3 The multiplex PCR primer used in this study

Bakteri target	Primer	Sekuens (5’ ke 3’)	Target site	Ukuran Produk (bp)
All *Lactobacillus*	IDL04F	AGGGTGAAGTCGTAACAAGTAGCC	1178–1198	-
All *Lactobacillus*	IDL03R	CCACCTTCCTCCGGTTTGTCA	1499–1522	-
L.acidophilus	IDL22R	AACTATCGCTTACGCTACCACTTTGC	2079–2104	606
L.plantarum	IDL62R	CTAGTGGTAACAGTTGATTAAAACTGC	1900–1926	428



(a) (b)

Figure 1 Electrophotogram of PCR product isolates *Lactobacillus spp*. from dangke: (a) cup sample 1 – 15; and (b) cup sample 16 – 30. Note. M = marker; and N = negative control

Discussion

Previously, several times the identification of lactic acid bacteria in dangke using conventional methods or culture methods has been carried out, so the results obtained are also limited. Whereas, milk and dairy products are nutrient-rich habitats for the growth of lactic acid bacteria, so that various species can be identified.

Sample preparation

A total of 30 samples of dangke were obtained from markets in 5 (five) sub-districts, namely Cendana District, Enrekang District, Anggeraja District, Alla District, and Baraka District, isolated lactic acid bacteria on MRSA + $CaCO_3$ 1% medium then incubated 2 x 24 hours at 37^oC and successfully identified 28 samples of lactic acid bacteria colonies growing marked by the presence of a clear zone in the medium and forming a single colony and the other 2 samples identified as not growing lactic acid bacteria colonies (Table 1). This is in accordance with what Nguyen et al. also used MRSA + 1% CaCO3 media when isolating lactic acid bacteria from fermented 'Nem chua' Vietnamese traditional food.[11] Lactic acid bacteria that grow on the medium will provide a clear zone around the colony after 2-3 days of incubation due to the production of lactic acid which will react with $CaCO_3$ to form Ca-lactate which is soluble in the medium.[4]

DNA extraction, PCR amplification, and Agarose gel electrophoresis

Species-specific primer novelty for the identification of *L.acidophilus* and *L. plantarum* were designed from 16S rRNA, 16S-23S rRNA intergenic spacer region, and 23S rRNA gene according to Kwon et al. and their sizes are in Table 3.[8]

This research shows that the identification of *Lactobacillus spp*. of the 30 dangke samples using multiplex PCR, only 14 samples were detected that displayed DNA banding patterns with details, 9 samples were detected containing *L. acidophilus* and 4 samples containing *L. plantarum*. This may be due to the presence of lactic acid bacteria other than *L. acidophilus* and *L. plantarum* in dangke (Table 2 and Figure 1). This is in line with previous research, lactic acid bacteria isolated from cow's milk dangke include species of *Streptococcus thermophilus, Lactobacillus lactis*, and *Lactobacillus thermophilus*[7], *Pediococcus pentosaceus*[5], *Lactobacillus fermentum* and *Lactobacillus*

acidophilus[14,1], *Lactococcus spp., Streptococcus spp., Leuconostoc spp., Pediococcus spp.*, and *Enterococcus spp.*[21], *Lactobacillus spp*.[15] and *Lactobacillus fermentum*[24]. Meanwhile, lactic acid bacteria isolated from buffalo's milk dangke include *Lactobacillus plantarum* and *Lactobacillus fermentum* species[13], and *Enterococcus faecium* DU55[19]. However, it cannot be used as an illustration for the entire population. This could be due to differences in identification methods and the number of species-specific primers used. Then it can be continued to the sequencing stage so that information is obtained about the diversity of LAB species found in dangke.

The advantage of using multiplex PCR in identifying lactic acid bacteria compared to other PCR methods is that it can amplify multiple targets in one PCR experiment. In multiplexing assays, more than one target sequence can be amplified using multiple primer pairs in one reaction mixture[6]. This is used to detect false negative results. The reaction can be said to be negative or fail if all the products do not appear on the visualization. Multiplex PCR is better able to indicate the quality of the DNA template than single PCR, as well as cost efficiency and preparation time when compared to single PCR[17,26]. Multiplex PCR is a technique that can be chosen if you want to spend money and use relatively few samples.

Conclusions

With the Multiplex PCR method, the highest yield was *Lactobacillus acidophilus* (33.33%) compared to *Lactobacillus plantarum* (16.67%) in dangke samples obtained from markets in 5 (five) sub-districts in Enrekang Regency, South Sulawesi.

Should be carry out further molecular identification using several primers from LAB species then proceed to the level of RAPD genotyping identification using specific primers at one particular locus or on a specific gene.

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