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RESEARCH ARTICLE
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Isolation and Characterization of Lactic Acid Bacteria Isolated from Traditional Dairy Product Dangke Cheese using Molecular 16s rRNA Gene Sequence PCR Method

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ABSTRACT

Dangke cheese is one of the typical fermented milk products from the Enrekang region of South Sulawesi, Indonesia. Dangke has acidic characteristics similar to other fermented milk products, which are thought to be produced by natural microorganisms called indigenous lactic acid bacteria (LAB) in milk. This study aims to isolate and identify lactic acid bacteria (LAB) from traditional Dangke milk products using a molecular method based on 16s rRNA gene sequencing. Lactic acid bacteria were isolated from Dangke by identifying macroscopically, microscopically, and biochemically. Then, molecular identification was performed using the PCR (Polymerase Chain Reaction) 16s rRNA gene sequencing method. The results showed that 15 LAB isolates were successfully isolated from Dangke, and molecular analysis revealed that six isolates were identified as *Enterococcus faecium*. This discovery provides important insights into the diversity of microorganisms in Dangke, especially the role of *Enterococcus faecium* as a potential lactic acid bacteria and underlines the value of Dangke as a source of local microorganisms with functional benefits that can support innovation in the food industry.

Keywords: Characterization, Dangke cheese, Isolation, Lactic acid bacteria, 16s rRNA gene sequence.

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INTRODUCTION

Lactic acid bacteria are a group of microorganisms widely used in the food industry, particularly in producing fermented dairy products such as cheese, yogurt, and kefir (Malaka et al., 2020). These bacteria are generally recognized as safe and have a long history of safe use in food production. They play a crucial role in developing flavor, texture, and consistency in various fermented food products (Kumar and Kumar, 2015).

Traditional cheese has long been part of the food culture in various countries, including Indonesia. One of Indonesia's typical fermented milk products is Dangke, which comes from the Enrekang area of South Sulawesi (Malaka et al., 2022). Dangke production has been ongoing since 1905 and has been passed down to the generation. The name "Dangke" is derived from the Dutch phrase "*dank u well*", which was used by the Dutch during colonization to express gratitude to the Enrekang citizen when they offered food (Yusuf et al., 2022). The Enrekang citizen shortened it to "Dangke", which became the name for this unique type of soft cheese and synonymous with the word "thank you" (Malaka et al., 2022). Dangke is made through a milk coagulation process involving the papain enzyme as a coagulant, without involving starter bacterial cultures as in cheese making in general (Melia et al., 2018). However, Dangke has acidic characteristics similar to other fermented milk products, which are thought to be produced by natural microorganisms called indigenous lactic acid bacteria (LAB) in milk (Syah et al., 2017).

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LAB are Gram-positive, catalase-negative microorganisms that efficiently metabolize carbohydrates into lactic acid under facultative anaerobic conditions, with no capacity for spore formation (Cai et al., 2024). LAB constitute a diverse group of bacteria that include the genera *Streptococcus, Lactococcus, Pediococcus, Enterococcus*, and *Lactobacillus,* which are predominantly found in dairy products and fermented foods (Akbulut et al., 2022). LAB are a group of microorganisms recognized for their capacity to convert carbohydrates into lactic acid, which not only imparts a characteristic flavor but also serves as a natural preservative, thereby enhancing the shelf life of food products (Anumudu et al., 2024).

In recent years, LAB have demonstrated significant contributions to the food industry, particularly in lactic acid production and probiotic application, while their microbial metabolic properties have garnered increasing scientific interest (Hakim et al., 2023; García-Ruiz et al., 2014).

LAB play an important role in milk fermentation by producing lactic acid through the glycolysis process, which not only provides a sour taste but also preserves the product by lowering the pH, thereby inhibiting the growth of pathogens (Mandha et al., 2022; Malaka et al., 2024). These bacteria are capable of breaking down complex macromolecules in food, including the degradation of indigestible polysaccharides and the modification of undesirable flavor compounds. Furthermore, LAB produce a range of metabolites during their metabolic processes, such as short-chain fatty acids, amines, bacteriocins, vitamins and exopolysaccharides (Wang et al., 2021; Liang et al., 2024). Several LAB strains are known to produce EPS, which has the potential for health because it has antitumor and anti-inflammatory activity and can boost the immune system (Patten and Laws, 2015).

Understanding the microbial diversity and characteristics of the lactic acid bacteria in this Dangke cheese can provide valuable insights into its production and potential health benefits (Djide et al., 2020). The identification of bacterial RNA from LAB serves as a precise method for obtaining isolate information from Dangke, offering greater accuracy compared to macroscopic, microscopic and biochemical characteristics (Zakariah et al., 2022). The rigorous identification of isolated strains involves the integration of physiological, biochemical, and molecular genetic approaches to ensure precise characterization (Urshev et al., 2024) 16S rRNA gene sequencing is a method for identifying LAB by analyzing their DNA. This technique compares the 16S rRNA gene sequence of a bacterium to know sequences in database (Rodríguez et al., 2022). Therefore, this study aims to isolate and characterize lactic acid bacteria from Dangke cheese by using 16s rRNA sequencing PCR method.

MATERIALS & METHODS

Sample Collection and Preparation

Dangke cheese samples were collected from the production of the Milk Processing Biotechnology Laboratory, Faculty of Animal Science, Hasanuddin University. This Dangke product was made using fresh cow's milk pasteurized at 65°C for 30 minutes with the addition of 0.2% papain enzyme and 0.3% salt. After the milk coagulated, it was filtered to separate the curd and whey. The filtered curd was then moulded using a Dangke mold prototype and stored in the refrigerator for 7 days for the ripening process.

Isolation of Lactic Acid Bacteria

Dangke samples were obtained from the production results of the Milk Processing Biotechnology Laboratory and then prepared by taking 10g of Dangke, suspended in 90mL of sterile physiological solution (0.9% NaCl) and homogenized. Then, 1mL was taken and put into a dilution tube containing 9mL of distilled water and homogenized to produce a dilution of 10-1. The dilution process was continued until the dilution was 10-3. Aliquots of 0.1mL from each dilution were spread on de Man, Rogosa, and Sharpe agar plates and incubated anaerobically at 37°C for 48 hours under anaerobic conditions to create an environment favorable for the growth of these fastidious microorganisms. The colonies that grew and formed around a clear zone were re-inoculated into new MRSA media for 48 hours to undergo a purification stage three times until a single colony was obtained. The pure isolate was then transferred to slant agar and MRSB as a stock isolate and stored in a refrigerator at a temperature of 4°C.

Characterization of Lactic Acid Bacteria

Macroscopic Characteristics

Macroscopic identification was carried out by directly observing the isolate growing on agar media, including the colony shape, edge shape, color, and surface shape (elevation) formed on the isolate.

Microscopic Characteristics

Microscopic observation was carried out by making a smear preparation of the bacterial isolate on a glass object, which was then fixed and stained using Gram staining. The slide was then observed under a microscope with a magnification of 100x to determine the shape and Gram reaction of the bacterial cells.

Biochemical Characteristics

The biochemical characteristics of the isolates were analyzed, including the catalase test, Triple Sugar Iron Agar (TSIA) test, indole and motility test, Simmon's citrate test, urea test, growth conditions at pH 4, 6,8, and 10, as well as carbohydrate fermentation tests using glucose, lactose, sucrose, maltose, and galactose. All the tests were determined following standard microbiological methods.

Confirmation Test using 16S rRNA Sequencing PCR Test

The final step in identifying lactic acid bacteria was confirmation through 16S rRNA sequencing. This method has been widely used to classify and identify bacteria at the genus and species level based on their genetic characteristics. The sequences obtained from the 16S rRNA gene sequencing of the isolates were compared with the sequences available in the NCBI GenBank database using the BLAST algorithm to determine the most closely related species.

Data Analysis

Descriptive analysis was used to analyze the data obtained from the macroscopic, microscopic, and biochemical characterization of the lactic acid bacteria isolated from Dangke cheese by comparing isolate characteristics using the profile matching method based on Bergey's Manual of Systematic Bacteriology. The molecular data 16S rRNA sequencing PCR Test was analyzed using BLAST from the NCBI GenBank database. Using the neighbor-joining method, multiple sequence alignments were examined using MEGA X software to generate a phylogenetic tree and assess similarities and connections among the sequences.

RESULTS & DISCUSSION

Isolation of Lactic acid Bacteria

Dangke, which has gone through a ripening process for 7 days, was then selected for bacterial isolation in search of Dangke lactic acid bacteria strains (indigenous LAB). Isolation of bacteria originating from Dangke was carried out using a growth medium, Man de Rogosa Agar (MRSA), with an incubation temperature of 37°C for 48 hours in a facultative anaerobic condition. The selected isolates had a clear zone and slime texture (ropy) on the growing colonies, indicating them as lactic acid bacteria (Fig. 1).

Lactic acid bacteria have a clear zone formation due to the lowering of the pH in the surrounding medium resulting from the production of lactic acid through their fermentative metabolism (Yuliana et al., 2023). Otherwise, the slime texture that forms on the growing colonies indicates that the isolates potentially produce exopolysaccharides (Malaka, 2021).

The selected isolates were then purified 3 times through repeated inoculation on the MRSA media to get a single colony. Fifteen pure isolates were isolated from Dangke and identified as LAB using macroscopic morphology, microscopic morphology and biochemical assays. Every isolate has the main characteristics of lactic acid bacteria. Lactic acid bacteria's main characteristics are Gram-positive cocci, non-spore-forming, catalase-negative and can grow under anaerobic conditions (Hakim et al., 2023).

Macroscopic Characteristics

The macroscopic characteristics of the 15 isolates evaluated showed that all isolates had round colonies, entire margin, convex elevation, smooth surface, and white to yellowish color (Table 1). Macroscopic characteristic variations among isolates can be seen from the size of the colonies, which range from small to medium.

Lactic acid bacteria usually produce white to yellowish pigmented colonies on MRS agar due to the production of acids (Putri et al., 2020). LAB observed in Zakariah et al. (2019) showed that LAB isolated from Dangke cheese has convex elevation, while the margin is in the form of an entire. The color of the colony was macroscopically yellowish and white. Sulmiyati et al. (2018) also reported the same observation based on the macroscopic identification of LAB isolated from milk-fermented product kefir, which has a rounded shape, convex elevation, and white color.

Table 1: Macroscopics characteristics of LAB isolated from Dangke cheese

Sampel code	Colony morphology			
	Shape	Elevation	Margin	Color
DCA1	Circular	Convex	Entire	White
DCA2	Circular	Convex	Entire	White
DCA3	Circular	Convex	Entire	White
DCA4	Circular	Convex	Entire	White
DCA5	Circular	Convex	Entire	White
DCB1	Circular	Convex	Entire	White
DCB2	Circular	Convex	Entire	White
DCB3	Circular	Convex	Entire	White
DCB4	Circular	Convex	Entire	White
DCB5	Circular	Convex	Entire	White
DCC1	Circular	Convex	Entire	White to yellowish
DCC2	Circular	Convex	Entire	White to yellowish
DCC3	Circular	Convex	Entire	White to yellowish
DCC4	Circular	Convex	Entire	White to yellowish
DCC5	Circular	Convex	Entire	White to yellowish

Microscopic Characteristics

The microscopic characteristics of the 15 isolates were identified by using Gram's staining method. All isolates were identified as Gram-positive and coccal-shaped (Fig. 2), a typical characteristic of lactic acid bacteria. Most of the isolates showed pairs or short chains, arranging cells. Lactic acid bacteria are usually Gram-positive, coccal, or rod-shaped (Aritonang et al., 2022). Gram-positive bacteria have a thick peptidoglycan layer that prevents the loss of the crystal violet dye used in the Gram staining (Fevria and Hartanto, 2018).

Fig. 1: The growth of LAB colonies in MRSA medium that was isolated from Dangke cheese is indicated by the presence of a clear zone and slime texture around the colony.

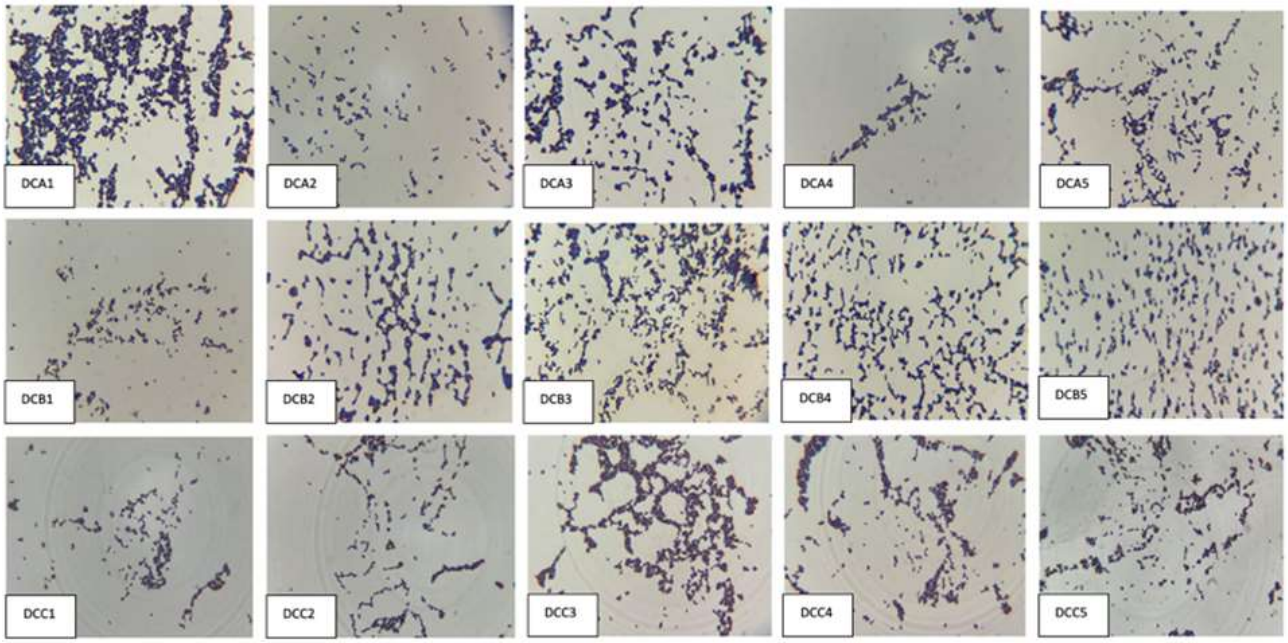

Fig. 2: Gram staining of lactic acid bacteria isolates from Dangke cheese at 100x magnification.

Table 2: Morphological and biochemical characteristics of bacteria isolate from Dangke cheese

Biochemical test	Isolate code														
	DCA1	DCA2	DCA3	DCA4	DCA5	DCB1	DCB2	DCB3	DCB4	DCB5	DCC1	DCC2	DCC3	DCC4	DCC5
Gram stain	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Shape	Coccus	Coccus	Coccus	Coccus	Coccus	Coccus	Coccus	Coccus	Coccus	Coccus	Coccus	Coccus	Coccus	Coccus	Coccus
Catalase	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
TSIA	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Gas	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Indol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Motility	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Temperature															
15°C	+	+	+	-	+	-	+	+	+	+	+	-	+	+	+
37°C	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
45°C	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
pH															
4	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
6	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
8	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
10	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
MR	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
VP	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Citrate	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Urea	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Glucose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Lactose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Sucrose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Maltose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Galactose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

(+): positive; (-): negative; TSIA: Triple Sugar Iron Agar; MR: Methyl Red; VP: Voges Proskauer.

Biochemical Characteristics

Biochemical tests, including TSIA, indol, motility, catalase, methyl-red, Voges-Proskauer, citrate utilization, carbohydrate fermentation, and growth at different pH and temperatures were conducted to confirm the identity of the isolates (Table 2). All isolates were catalase-negative, indicating that they do not produce catalase enzymes. This result is consistent with the typical characteristics of lactic acid bacteria, which are generally catalase-negative (Mokoena, 2017).

Triple sugar iron agar test showed positive results for acid production but negative for gas production. Indol and motility tests showed negative results for all isolates, indicating that the isolates were non-motile and did not produce indole. This indicating all the isolates were homofermentative lactic acid bacteria that produce only lactic acid as the main end product from carbohydrate fermentation, which are characteristic traits of lactic acid bacteria (Jafari et al., 2021; Siburian et al., 2021).

The methyl-red test showed positive results, meaning that the isolates were able to produce sufficient acid from glucose fermentation. The Voges-Proskauer test showed negative results, indicating that the isolates did not produce acetoin as the end product of glucose metabolism (Shanmugaraj et al., 2021; Ngouénam et al., 2024).

The citrate utilization test showed a negative result. The isolates did not show any growth or color change, suggesting that they could not use citrate as the sole

carbon source for their metabolism (Khushboo et al., 2023). The urea test showed a negative result, indicating that the isolates could not hydrolyze urea. Urea hydrolysis is not common among lactic acid bacteria (Hafsan and Mustami, 2015).

Carbohydrate fermentation tests showed that all isolates could ferment glucose, lactose, sucrose, maltose, and galactose. The ability to ferment these carbohydrates is a typical characteristic of lactic acid bacteria. Based on their ability to ferment glucose, the lactic acid bacteria could be divided into homofermentative and heterofermentative bacteria. The heterofermentative LAB could produce gas as an end product of fermentation, while the homofermentative LAB did not produce gas on fermentation (Syah et al., 2017).

The growth at different pH showed that all the isolates could grow at pH 4, 6, and 8 but not at pH 10. This indicates that the isolates are acid-tolerant and can grow in acidic environments, a common feature of lactic acid bacteria. Growth of the isolates at low pH levels suggests that they possess an intrinsic acid tolerance, a trait frequently observed in lactic acid bacteria (Nofiani et al., 2022). Microorganisms utilized as probiotics must meet certain criteria in order to survive in the intestinal tract and demonstrate their useful features and health advantages. The most crucial feature is their capacity to withstand harsh gastrointestinal conditions, particularly acid and bile stress and subsequent colonization in the digestive tract (Farhangfar et al., 2021).

The growth at different temperatures showed that all isolates could grow at 15, 37 and 45°C, indicating the isolates are mesophilic bacteria. Microorganisms could grow at different temperature ranges, namely psychrophilic (4-15°C), mesophilic (20-45°C) and thermophilic (40-60°C) (Sudiartha et al., 2023).

Based on the macroscopic, microscopic, and biochemical characterization results, the 15 isolates obtained from Dangke were identified as lactic acid bacteria. Based on matching profile bacteria in Bergey's Manual of Systematic Bacteriology (Whitman, 2009), they most likely belong to Enterococcus or Lactococcus genus. This genus has been widely reported as a predominant LAB group in various dairy products like Dangke.

16S rRNA Sequencing PCR Analysis

Isolates were further subjected to 16S rRNA gene sequencing to achieve more accurate identification at the species level. 6 isolate samples were successfully amplified using 16S rRNA gene sequencing by PCR analysis. All the isolates were stretched and identified at 1500bp (Fig. 3). Furthermore, a phylogenetic tree revealed that all six isolates belonged to the *Enterococcus faecium* group, and the tree is primarily made of four clusters (Fig. 4).

A bootstrap value of 99 indicates that the isolates are very closely related. This could mean they come from the same source or environment or have similar evolutions. The isolates from the Dangke samples (DCA2, DCA3, DCB3, DCB5, DCC1, DCC3) are closer to strain groups such as *Enterococcus faecium strain HBUSA562391,* but still form their own branch. This suggests an evolutionary relationship, but the isolates from the Dangke sample are genetically different enough to create a unique group. This phylogenetic tree is usually constructed using molecular analysis based on a specific gene. The 16S rRNA gene is often used in bacterial phylogenetic studies because of its high conservation, making it suitable for determining phylogenetic relationships. Isolates DCA, DCB, and DCC form a clear cluster with a high bootstrap value of 99. This indicates that the isolates have very high genetic similarity because they come from the same Dangke fermentation products.

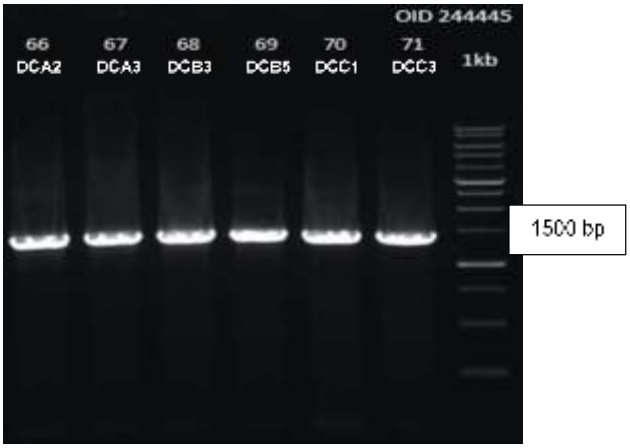

Fig. 3: 16s rRNA amplified by PCR with 1% agarose gel electrophoresis.

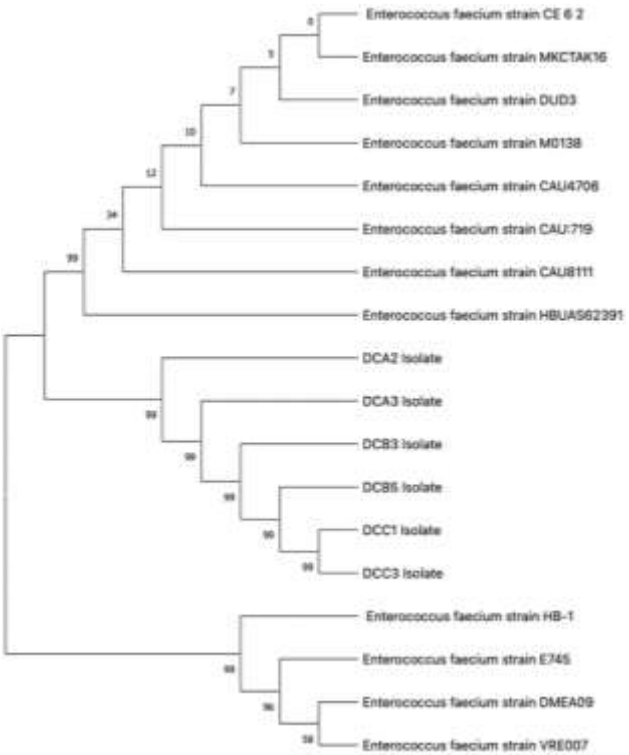

Fig. 4: Phylogenetic tree based on the analysis of PCR using the 16S rRNA gene sequencing method and the nearest isolate, revealing the phylogenetic location of the representative strain isolated from Dangke.

In this study, from entering isolation, identification, and characterization, the lactic acid bacterial isolates obtained from Dangke cheese were identified as belonging to the genus *Enterococcus*, and based on the 16S rRNA gene analysis found that the isolates are likely *Enterococcus faecium*. Identifying *Enterococcus faecium* as

the predominant LAB in Dangke samples is consistent with previous studies on the microbial ecology of traditional fermented dairy products (Hakim et al., 2023). The ability of this species to grow and dominate in the Dangke environment is likely due to its inherent acid tolerance and adaptability to cheese-making conditions.

Enterococcus faecium is an important lactic acid bacterium widely reported in various fermented dairy products, especially cheese products (Dapkevicius et al., 2021). *Enterococcus faecium* is a Gram-positive bacterium from the Lactobacillus family, and it is the third-largest genus after *Lactobacillus* and *Streptococcus*. They are gram-positive, catalase- and oxidase-negative, non-spore-forming, facultative anaerobic cocci that appear either as single bacteria, in pairs, short chains, or groups (Terzić-Vidojević et al., 2021; Han et al., 2023). Its characteristics allow it to survive and grow well in acidic environments, such as those found in fermented milk products like Dangke cheese. *Enterococcus faecium,* as a lactic acid bacterium, plays a crucial role in the fermentation process, contributing to the development of flavor, texture and preservation of this dairy product (Widyastuti and Febrisiantosa, 2014).

Enterococcus spp. is one of the LAB species found in dairy products and can survive in several environments (Amaral et al., 2017). Other *Enterococcus* strains have been demonstrated to offer health benefits. These strains have numerous antibiotic susceptibility and virulence characteristics, which are thought to contribute to their beneficial features (Wu et al., 2022).

Several studies have shown that these strains have high health-promoting properties, such as immune response stimulation, anti-inflammatory activity, hypocholesterolemic action and use in the prevention/treatment of specific diseases (Jia et al., 2014; Terzić-Vidojević et al., 2021; Yerlikaya et al., 2021). These bacteria produce a variety of bioactive substances, including enterocins, which are antimicrobial compounds that can diminish or inhibit a wide range of pathogens, organic acids, selenoproteins, and exopolysaccharides (Zanzan et al., 2023).

Conclusion

A total of 15 Isolates from traditional Dangke cheese were successfully isolated. The 16s rRNA gene sequencing method analysis showed that as many as six isolates were successfully identified as *Enterococcus faecium*. *Enterococcus faecium* isolated from Dangke shows interesting potential for further research, especially in evaluating its ability to produce exopolysaccharides. These findings provide new insights into microorganisms in traditional foods that can be utilized for biotechnology applications, especially in developing functional food products.

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Author's Contribution: RWK and RM: Conceived and designed the experiments, conducting field experiments, analyzing data and writing paper; RM, NN, WW: supervised, analyzing data and writing paper; FAA: conducted lab analysis and writing paper. All authors read and approved the final manuscript.

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