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Characterization of Lactic Acid Bacteria Isolated from Gambir with Potential Probiotic Properties

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ABSTRACT

Gambir is commonly used as a key ingredient in betel quid and is one of Indonesia's major agricultural commodities. West Sumatra is the primary production region, contributing approximately 80-90% of the country's total gambir production. This study aimed to investigate the potential of lactic acid bacteria (LAB) isolated from gambir, assessing their potential probiotic qualities using biochemical and molecular approaches. Biochemical characterization included catalase activity testing and fermentation pattern analysis, while molecular identification was carried out through the *16S rRNA* gene sequence. The results revealed that the isolated LAB strains were Gram-positive, bacilli-shaped, catalase-negative, and exhibited hetero-fermentative behavior. Further biochemical analysis confirmed their ability to ferment a variety of sugars but not produce gas from glucose. Basic Local Alignment Search Tool analysis showed that the bacterial isolate from gambir, labeled with the sample code GM2, was closely related to *Lactiplantibacillus pentosus*, a species recognized for its probiotic potential. The isolates showed antimicrobial activity against common foodborne pathogens like *Salmonella* spp. and other pathogens, suggesting their potential use in food preservation. The present study also demonstrated the LAB isolates' tolerance to low pH and bile salts, which are key attributes for probiotic candidates. Thus, the findings of the current study suggest that LAB from gambir possess promising characteristics for application in probiotic products and as biocontrol agents in food safety.

Keywords: *16s rRNA*, Gambir, Lactic acid bacteria, Probiotic

INTRODUCTION

Gambir is widely recognized as a key ingredient in betel quid and stands out as one of Indonesia's top agricultural commodities. West Sumatra plays a dominant role in its production, accounting for approximately 80-90% of the country's total gambir output. Within this region, Lima Puluh Kota Regency is the largest producer, contributing 70.39% of the total production (Nasution et al., 2015). In addition to its use in betel quid, gambir has applications in various industries, including batik, paint, and textiles, it is derived from the sap of the leaves and twigs of the *Uncaria gambir* plant, which is then processed and dried to produce the final gambir product (Aditya and Arianti, 2016). In Sumatra, Gambir is known by various local names, including gambee, gani, kacu, sontang, gambe, gambie, gambu, gimber, pengilom, and selet (Anggraini et al., 2011).

This plant has a subtle odor and a taste that begins as bitter and astringent but gradually becomes mildly sweet. Gambir is also recognized as a valuable source of antioxidants and belongs to the Rubiaceae family (coffee family). It contains several polyphenolic compounds, such as catechins (catechin acid), catechin tannate, and quercetin (Ndagijimana, 2013; Firdausni et al., 2020). These compounds have well-documented antioxidant properties and may play a crucial role in supporting the growth and viability of lactic acid bacteria (LAB). Specifically, catechins and other polyphenols have been shown to exert antimicrobial effects, selectively inhibiting the growth of harmful pathogens while potentially fostering the growth of beneficial LAB (Choe and David, 2009). Furthermore, these antioxidants can enhance LAB stress tolerance, protecting them from oxidative damage and improving their survival under challenging conditions, such as low pH and bile exposure (Choe and David, 2009). Thus, the bioactive compounds in gambir may contribute significantly to the effective isolation of LAB strains with potential probiotic properties, highlighting gambir as a promising natural source for probiotic applications. Gambir (*Uncaria gambir*) may possess several unique properties that make it a promising candidate for the isolation of LAB, particularly for probiotic applications. These properties include: rich polyphenolic content, antioxidant properties, natural antimicrobial activity, source of nutrients for lab growth, and

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potential prebiotic effect. This evaluation encompasses morphological identification, biochemical profiling, molecular DNA analysis, and purification to evaluate the probiotic potential of the subject for promoting overall health (Syukur et al., 2014). The LAB have been recognized for producing lactic acid, hydrogen peroxide, and carbon dioxide and for breaking down food into simpler components, such as glucose, fructose, amino acids, and short-chain fatty acids (Stanton et al., 2001).

Among the probiotics, LAB can inhibit and eliminate pathogenic bacteria from their surroundings (Trisna and Wahud, 2012). They are classified as safe microorganisms (food-grade microorganisms) and are generally recognized as safe (GRAS) for health. Probiotics, as live microorganisms, have been shown to provide beneficial effects by balancing the gut microbiota (Trisna and Wahud, 2012). Gambir has been used traditionally in Southeast Asia for its astringent. The astringent and anti-inflammatory properties of gambir could indirectly influence gut health by reducing inflammation in the digestive system and promoting overall digestive balance, which could create conditions favorable for LAB (Melia et al., 2015). The benefits of consuming probiotics include maintaining the balance of microorganisms in the digestive system (Dewi et al., 2021). A study demonstrated that supplementing *Lactobacillus bulgaricus* in both the feed and drinking water of quails resulted in positive effects on live weight, feed intake, and feed conversion ratio (FCR) (Vahdatpour and Babazadeh, 2016). Kusumawati (2002) outlines several beneficial health effects of probiotics, including enhancing immune defense against infectious diseases, particularly gastrointestinal infections, and diarrhea, lowering blood serum cholesterol levels, alleviating symptoms of lactose intolerance, exhibiting antihypertensive properties, supporting digestive processes, possessing anti-mutagenic and anti-carcinogenic activities, influencing immune system responses, and decreasing the risk of colon cancer and tumor development. Isolating and identifying LAB from gambir is crucial to determining and obtaining LAB species with potential probiotic properties, particularly concerning their ability to promote gut health by balancing the microbiota, inhibiting pathogenic bacteria, and providing beneficial health effects, such as anti-inflammatory, antimicrobial, and immune-modulating activities.

MATERIALS AND METHODS

Study design

This experimental laboratory research was designed to isolate and characterize LAB from gambir samples. The study was conducted at the Microbiology Laboratory, Department of Animal Product Technology, Faculty of Animal Science, Andalas University.

Sampling

The gambir sample taken is gambir sap that has been fermented or dried. Three different gambir samples were used in this study, labeled GM1, GM2, and GM3, which were sourced from different locations in Limapuluh Kota Regency, West Sumatra, Indonesia. The samples were stored at 4°C (refrigerated) in sterile containers to prevent the growth of unwanted microorganisms and maintain the integrity of the sample. All storage procedures followed standard microbiological protocols to prevent cross-contamination and preserve sample quality.

Isolation and identification of lactic acid bacteria

The bacterial isolates from Gambir were identified based on the guidelines of Purwati et al. (2005). A one-gram portion of the sample was mixed with 9 mL of MRS Broth in a test tube and vortexed until the mixture was fully homogenized, referred to as the 10^{-1} dilution. From this, 100 µL was transferred into an Eppendorf tube containing 900 µL of fresh MRS Broth, followed by vortexing to achieve uniformity, representing the 10^{-2} dilution. This serial dilution was carried out until the 10^{-6}dilution was obtained. For the 10^{-6}dilution, 100 µL of the solution was spread onto MRS agar plates using the spread plate method. The inoculum was evenly distributed using a sterile hockey stick. The plates were then placed in an anaerobic jar and incubated at 37°C for 48 hours.

Gram staining

A bacterial sample was spread onto a clean, alcohol-disinfected glass slide and allowed to air dry over a Bunsen burner or drying apparatus. A drop of crystal violet was applied for one minute, followed by rinsing with distilled water and drying again. Afterward, iodine was added for one minute, and the slide was rinsed and dried once more. The slide was briefly immersed in diluted alcohol to wash off the excess stain and then treated with safranin for 30 seconds. Following another rinse with distilled water, the slide was air-dried over the Bunsen flame before being examined under a microscope (Romadhon et al., 2012).

Catalase test

To perform the catalase test, 10 µL of the LAB isolate was placed on a clean glass slide. A 10 µL drop of 3% hydrogen peroxide (H_2O_2) was added, and the formation of bubbles was observed to detect the presence of catalase activity (Public Health England, 2014).

Fermentation type test for lactic acid bacteria

The LAB isolate was inoculated in 5 mL of MRS Broth (Neogen Culture Media), and an inverted Durham tube was placed in the test tube. After incubating at 37°C for 48 hours, the presence of gas bubbles in the Durham tube was recorded to determine the type of fermentation (Romadhon et al., 2012).

Acid tolerance test

The acid tolerance test followed the method of Sunaryanto and Marwoto (2013) to obtain LAB isolates with high tolerance to gastric conditions (pH 3). A 0.1 ml bacterial suspension from MRS Broth was added to a series of tubes containing 9 ml of sterile MRS Broth (Merck, Germany) and incubated at 37°C for 90 minutes. The pH was adjusted by adding HCl 5 N. The cultures were then grown on MRS agar for 24 hours at 37°C.

Bile salt tolerance test

Bile salt tolerance testing of LAB was carried out using the method of Sunaryanto and Marwoto (2013). The MRS Broth (Merck, Germany), which contains 0.3% and 0.5% oxgall, was inoculated with one ml of the test bacterial culture and incubated at 37°C for 5 hours. Then, 100 µl of the culture was transferred into 900 µl of MRS Broth in Eppendorf tubes for enrichment. The culture was subsequently grown on MRS agar for 24 hours at 37°C. The number of surviving bacteria was counted using the plate count method. Viability (%) was used to compare the total number of cells before and after incubation. Bacteria with higher viability percentages after bile salt exposure were considered more likely to survive.

Antimicrobial activity

The antimicrobial activity of LAB isolates was evaluated against *Escherichia coli*, *Staphylococcus aureus*, and *Listeria monocytogenes*. The tests were conducted in triplicate for accuracy. Ampicillin and kanamycin, both well-known broad-spectrum antibiotics, were used as positive controls due to their established antimicrobial properties. The LAB cultures were grown in MRS Broth for 18-22 hours. Fresh overnight cultures of the target bacterial pathogens were inoculated into 20 mL of nutrient agar at a final concentration of 0.2% (v/v). After the MRS agar solidified, wells with a diameter of 6 mm were created. Subsequently, 50 µL of the LAB supernatant was introduced into each well. The plates were incubated anaerobically at 37°C for 14-16 hours, and the inhibition zones were measured to assess antimicrobial activity (Kaushik et al., 2009).

Molecular identification

DNA extraction from LAB was carried out using a commercial kit (Promega, USA). Polymerase Chain Reaction (PCR) was performed with the forward primer F 16S-27F (5'-AGA GTT TGA TCC TGG CTC AG-3') and the reverse primer R 16S-1492R (5'-GTT TAC CTT GTT ACG ACTT-3'), following the protocol in the Table 1 provided by Promega (2010).

Molecular applications for analyzing bacterial diversity through *16S rRNA* gene analysis are increasingly used to overcome challenges in bacterial cultivation. The *16S rRNA* gene is particularly suitable for bacterial identification because it is present in all organisms (Suryani et al., 2010). Chun et al. (2007) further emphasized that the use of *16S rRNA* gene sequences for studying the phylogeny of bacteria has become widespread. The function of the *16S rRNA* gene remains relatively unchanged over time (mutations), suggesting that random sequence changes provide a more accurate measure of evolutionary time. Additionally, the *16S rRNA* gene, which is approximately 1,500 base pairs long, is sufficiently large to serve as a valuable tool for bioinformatics purposes. The *16S rRNA* gene sequence has been extensively used for prokaryotic organism identification. Sequence comparisons based on nucleotide similarity values and phylogenetic methods are effective for identifying bacterial isolates.

Table 1. Polymerase chain reaction program for *16S rRNA* gene

Process sequence	Temperature	Time
Pre-denaturation	95°C	2 minutes
Denaturation	95°C	45 seconds
Annealing	56°C	45 seconds
Extension	72°C	1 minute and 40 seconds
Final Extension	72°C	10 minutes
Cooling	4°C	~ (until completion)

Data analysis

Sequencing data were processed using the BioEdit software for analysis. To perform sequence alignment, the obtained sequences (query) were compared to those stored in GenBank by conducting database searches on the NCBI website using the BLAST Tool (Depson, 2012).

RESULTS AND DISCUSSION

Isolation of lactic acid bacteria

The total LAB in Table 2 count in gambir samples GM1, GM2, and GM3 were 48×10^8 CFU/g, 52×10^8 CFU/g, and 50×10^8 CFU/g, respectively. These results aligned with other studies using similar raw materials, such as *gulai paluik*. The total LAB colonies in this study were higher than those reported by Meriza (2021) and Pratama (2020), who found 11×10^7 CFU/g and 25×10^7 CFU/g, respectively. This higher count was attributed to the fact that gambir is a fermented product, which typically results in a higher LAB colony count. Based on these results, the LAB colonies identified in this study can be categorized as probiotic candidates, consistent with Wijayanto's (2009) assertion that probiotic bacterial cell viability should range between 10^7–10^9 CFU/g.

Table 2. Total colonies of lactic acid bacteria isolated from Gambir

Sample code	Total lactic acid bacteria ($\times10^8$ CFU/g)
GM1	48
GM2	52
GM3	50

Identification of lactic acid bacteria

Macroscopic observations in Table 3 reveal LAB isolates with characteristics including round shape, smooth texture, creamy color, shiny convex surface, and smooth, regular colony edges on MRS agar. This finding was consistent with the findings of Purwati et al. (2005), who reported similar colony characteristics for LAB on MRS agar. Additionally, LAB isolates from soursop fruit by Delfahedah et al. (2013) and from fermented *pisang kepok* (a type of banana) by Finanda et al. (2021) showed round, smooth-edged, convex colonies with milky white and creamy colors. The LAB in Table 4 isolates were catalase-negative, as evidenced by the absence of air bubbles caused by O^2 gas. LAB did not produce the catalase enzyme, which breaks down H^2O^2 into water and non-harmful oxygen. To convert hydrogen peroxide into oxygen, an enzyme called catalase is needed (Carroll et al., 2017). However, LAB isolated in this study could not produce this enzyme.

Table 3. Morphological characteristics of the lactic acid bacteria isolate

Sample	Colony form	Color	Size	Surface	Corner
GM1	Bacilli	White-beige	1.5 mm	Slippery, convex	Flat, smooth
GM2	Bacilli	White-beige	2.0 mm	Slippery, convex	Flat, smooth
GM3	Bacilli	White-beige	1.5 mm	Slippery, convex	Flat, smooth

Table 4. Biochemical properties of the lactic acid bacteria gambir isolate

Sample	Catalase test	Fermentation type
GM1	Negative	Hetero-fermentative
GM2	Negative	Hetero-fermentative
GM3	Negative	Hetero-fermentative

Resistance of lactic acid bacteria to stomach acid

A critical quality for probiotics was their survival in the acidic environment of the stomach, which was assessed by testing LAB resistance to low pH. It was essential to select probiotic strains that thrived in acidic conditions to maximize their efficacy.

Based on Table 5, the acidity of the stomach served as the first gate for selecting bacteria before they entered the intestines. The viability results of LAB under acidic conditions (pH 3) ranged from 66.8% to 88.5%. Higher viability levels indicated that the bacteria were more resilient to low pH conditions. The GM2 isolate showed the highest viability at 88.5%. Each isolate displayed varying viability due to differences in its ability to tolerate the acidic conditions of the stomach. Wijayanto (2009) observed that most microorganisms typically undergo cell death in highly acidic

environments or at low pH levels. This is because extreme acidity can damage cell membranes, causing the release of intracellular components, such as magnesium, potassium, and lipids, ultimately leading to cell death. Furthermore, resistance to stomach acid is a critical criterion for an isolate to be considered a probiotic (Khuruna and Kanawijaya, 2007).

Resistance of lactic acid bacteria to bile salts

This test was conducted to evaluate the survival ability of gambir LAB isolates in the intestinal environment, which contains a bile salt concentration of 0.3%.

Based on Table 6, the viability of LAB against bile salts ranged from 70.3% to 92.4%. The LAB isolate GM2 exhibited the highest viability among the other isolates. Higher percentages indicated greater resistance of LAB to bile salts. Leverrier et al. (2003) noted that bile salts could cause morphological changes in bacterial cells due to protein release from the cells. Wijayanto (2009) stated that the viability of bacterial cells, which were candidate probiotics, should range between 10^6 and 10^9 CFU/mL to survive in the gastrointestinal tract.

Antimicrobial activity

The highest viability of LAB against acid and bile salts was observed in LAB GM2, which was subsequently evaluated for its antimicrobial activity against pathogenic bacteria. Isolate LAB GM2 demonstrated the ability to inhibit pathogenic bacteria.

Based on Table 7, the inhibition zone for *E. coli* O157 measured 9.56 mm, which was comparable to the findings of Meriza (2021) regarding the antimicrobial activity of LAB isolates from *gulai paluik* against *E. coli* O157, which showed an inhibition zone of 11.51 mm. The inhibition zone diameter for *S. aureus* was 10.83 mm, while for *L. monocytogenes*, it measured 6.18 mm. According to Pelczar and Chan (2008), a larger clear zone indicates stronger inhibitory activity of LAB isolates against pathogenic bacteria. The inhibition activity can be categorized into four levels: weak activity (< 5 mm), moderate activity (5–10 mm), strong activity (10–20 mm), and very strong activity (> 20-30 mm, Morales et al., 2003).

Table 5. Resistance test of lactic acid bacteria isolated from gambir to acid pH

LAB isolate sample	Number of bacterial cells (CFU/ml) $\times 10^8$		LAB viability (%)
	Control	pH 3	
GM1	23.3	19.5	83.7
GM2	16.5	14.6	88.5
GM3	16.0	10.7	66.8

CFU: Colony forming unit, LAB: Lactic acid bacteria

Table 6. Test of the resistance of lactic acid bacteria isolated from gambir to bile salts

LAB isolate sample	Number of bacterial cells (CFU/ml) $\times 10^8$		LAB viability (%)
	Control	Oxgall 0.3%	
GM1	9.4	8.1	86.2
GM2	10.6	9.8	92.4
GM3	15.5	10.9	70.3

CFU: Colony forming unit, LAB: Lactic acid bacteria

Table 7. Clear zone diameter test antimicrobial activity test of GM2 isolate with antibiotics as a positive control

Sample code	Diameter of clear zone (mm)		
	E. coli O157	*S. aureus*	*L. monocytogenes*
GM2	9.56	10.83	6.18
Ampicillin	12.36	12.22	10.23
Kanamycin	15.23	13.14	11.08

E. coli O157: *Escherichia coli* O157; *S. aureus*: *Staphylococcus aureus*; *L. monocytogenes*: *Listeria monocytogenes*

Molecular identification

The sequencing results of the LAB isolated from Gambir, sample code GM2, were compared with Gene Bank data using the BLAST program on the NCBI website, as shown in Figure 1.

Based on Figure 2, the analysis results using BLAST, the LAB isolates from *gambir* GM2 had 99.33% similarity with *Lactiplantibacillus pentosus*, with a sequence length of 1492 bp. Hagstrom et al. (2000) suggested that isolates with

16S rRNA sequence similarity greater than 97% could represent the same species. In contrast, sequence similarities between 93-97% could represent bacterial identities at the genus level but differed at the species level. BLAST analysis showed that the bacterial isolate from gambir, labeled with the sample code GM2, was closely related to *Lactiplantibacillus pentosus*.

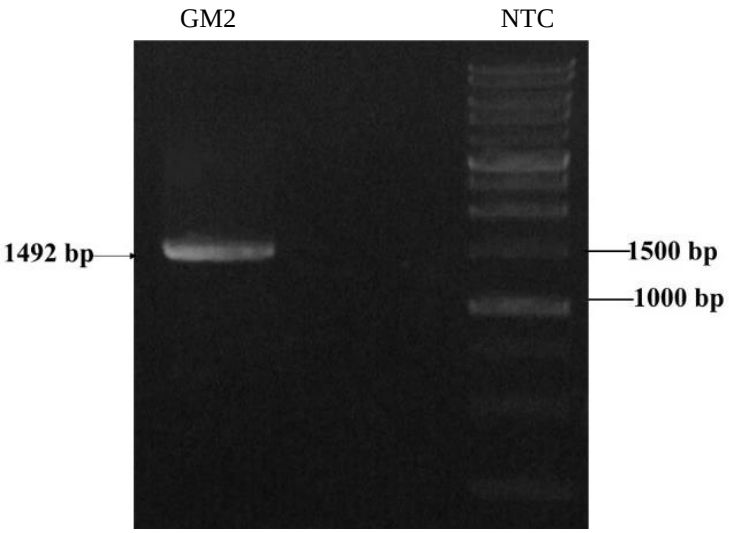

Figure 1. Results of electrophoresis of lactic acid bacteria gambir isolate. GM2: Gambier sample, NTC: Control without template

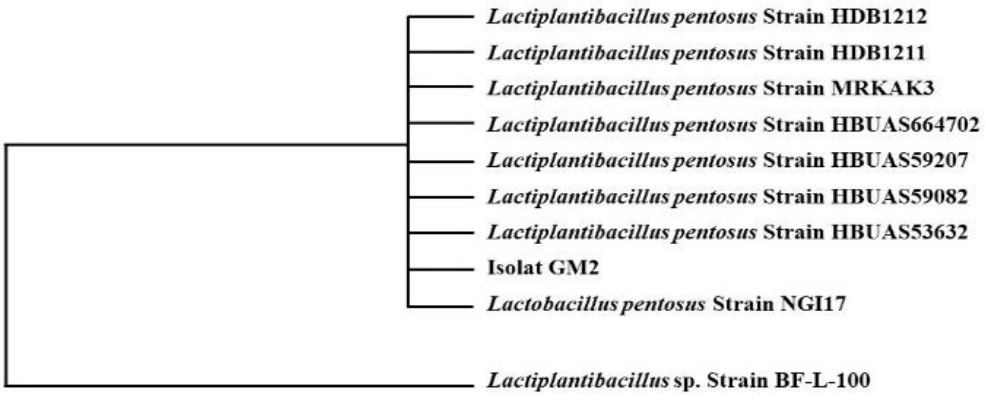

Figure 2. Phylogenetic tree of lactic acid bacteria GM2 isolated from Gambir. GM2: Gambir isolate

CONCLUSION

The LAB B isolated from gambir with sample code GM2 exhibited a bacillus morphology, white in color, and it was classified as a gram-positive bacterium. The isolate of GM2 was catalase-negative, demonstrated hetero-fermentative fermentation, and showed acceptable resistance to stomach acid and bile salts. Molecular identification confirmed that LAB GM2 was *Lactiplantibacillus pentosus*. It is recommended that future research be conducted *in vitro*, *in vivo*, and in clinical studies to evaluate the probiotic effects of *Lactiplantibacillus pentosus* GM2.

DECLARATIONS

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Authors' contributions

Varhanno Khallifhatul Khanh was responsible for the overall design and execution of the research, data collection, and analysis. Writing the manuscript and ensuring all necessary revisions were made by all authors. Muthia Sukma assisted in the conceptualization of the study and contributed to the experimental design. Additionally, Muthia Sukma

helped with data analysis and interpretation. Rahmi Fithria contributed to the literature review, interpretation of results, and final editing of the manuscript. All authors have read and approved the final version of the manuscript before publication in the present journal.

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Competing interests

The authors have not declared any conflict of interest.

Ethical considerations

This paper was originally written by the authors and has not been published elsewhere. The authors checked the text of the article for plagiarism index and confirmed that the text of the article is written based on their original scientific results.

Availability of data and materials

The data to support this study finding is available upon reasonable request to the corresponding author.

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