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A Polyphasic Approach to the Exploration of Lactic Acid Bacteria from Tempoyak in South Sumatra

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ABSTRAK

Pendekatan polifasik merupakan metode yang sangat komprehensif untuk mengklasifikasikan isolat bakteri ke dalam taksa tertentu. Pendekatan ini mencakup informasi lengkap yang diperoleh dari karakter morfologi dan biokimia, serta dilengkapi dengan informasi molekuler seperti pengurutan gen 16S rRNA. Penelitian ini bertujuan untuk mengidentifikasi spesies BAL (bakteri Asam Laktat) yang terdapat pada tempoyak dari Sumatera Selatan. Identifikasi BAL dilakukan dengan mengamati karakter morfologi koloni, morfologi sel, fisiologi biokimia serta analisis gen 16S rRNA BAL. Karakter fisiologi dan biokimia BAL diamati melalui uji kebutuhan oksigen, uji motilitas, uji indol, uji katalase, uji MR-VP, uji sitrat, uji urea, dan uji fermentasi gula. Karakter morfologi, biokimia dan fisiologi dibandingkan dengan Bergey's Manual of determinative Bacteriology. Gen 16S rRNA diamplifikasi menggunakan primer universal 27F (5' AGA GTT TGA TCM TGG CTC AG 3') dan 1492R (5' TAC GGY TAC CTT GTT ACG ACT T 3'). Urutan DNA dalam format FASTA dibandingkan dengan database DNA di NCBI melalui software BLAST. Pohon filogenetik isolat BAL yang diperoleh dikonstruksi menggunakan metode Maximum likelihood pada MEGA X dengan bootstrap 1000. Penelitian ini berhasil mengisolasi tiga isolat Bakteri Asam Laktat (BAL) yang diberi kode TP02, TP07 dan TP08. Berdasarkan pendekatan polifasik, TP02 terindentifikasi sebagai Lactobacillus sp, YIT 11469, TP07 teridentifikasi sebagai Lentilactobacilus kefiri strain NBRC 15888 dan TP08 teridentifikasi sebagai Lentilactobacillus buchneri strain JCM 1115. TP02 dan TP08 tervalidasi memiliki kasamaan pada tingkat strain dengan persentase identitas masing-masing sebesar 99,93% dan 99,60%.

ABSTRACT

The polyphasic approach is a comprehensive method for classifying bacterial isolates into specific. It combines morphological and biochemical data with molecular details, such as 16S rRNA gene sequencing. This study aims to identify LAB (Lactic Acid Bacteria) species found in tempoyak from South Sumatra. The identification process includes examining colony morphology, cell morphology, physiology biochemistry, and analyzing the 16S rRNA gene of LAB. The physiological and biochemical characteristics of LAB were observed through test such as oxygen requirement, motility, indole, catalase, MR-VP, citrate, urea, and sugar fermentation. The Morphological, biochemical and physiological characteristics were compared to those in Bergey's Manual of Determinative Bacteriology. The 16S rRNA gene was amplified using universal primers 27F (5' AGA GTT TGA TCM TGG CTC AG 3') and 1492R (5' TAC GGY TAC CTT GTT ACG ACT T 3'). DNA sequences in FASTA format were compared to NCBI DNA database using BLAST software. A Phylogenetic tree was reconstructed using the Maximum likelihood method in MEGA X with a bootstrap of 1000. This study successfully isolated three LAB, coded as TP02, TP07 and TP08. TP02 was identified as Lactobacillus sp, YIT 11469, TP07 as Lentilactobacilus kefiri strain NBRC 15888 and TP08 as Lentilactobacillus buchneri strain JCM 1115. TP02 and TP08 showed strain level similarity with identity percentages of 99.93% and 99.60%, respectively.

1. INTRODUCTION

Mercury In recent decades, probiotic food products have become essential in the human diet. Probiotic foods are fermented foods containing live microorganisms that provide health benefits (Mohkam et al., 2022). Due to their potential health benefits, probiotic foods now dominate 60-70% of the functional food market (Ibrahim et al., 2023). These products contain live microorganisms that colonize the host's body, providing beneficial effects without being toxic or pathogenic (Mohkam et al., 2019). Lactic Acid Bacteria (LAB) are among the main microorganisms found in many fermented foods, and they play a key role in balancing. In South Sumatra, *tempoyak* is a well-known probiotic food product. It is highly nutritious, containing carbohydrates, protein, fat, vitamin B1, niacin, sodium, calcium, phosphorus, iron, potassium, vitamin B2, vitamin C and carotenoids. This rich nutritional profile makes *tempoyak* a commercial probiotic food (Anggadhania et al., 2023). The fermentation process of tempoyak occurs anaerobically, involving LAB (Arina et al., 2014). Various LAB strains are present in tempoyak, including

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Lactobacilus plantarum and *Lactobacilus fermentum* (Reli et al., 2017). Research by Erfisa et al. (2022) identified four species of microflora in *tempoyak*: *P. acidilactici, L. plantarum, L. curvatus,* and *Leu. Mesentroides,* which play a positive role in tempoyak fermentation. LAB identified so far belong to the phylum *Firmicutes*, class *Bacilli* and order *Lactobacillales,* with known families including *Aerococcaceae, Enterococcaceae, Carnobacteriaceae, Leuconostocaceae, Streptococcaceae*, and *Lactobacillaceae* (Hasbi et al., 2024). Certain LAB strains are used as probiotics, either in pure or mixed cultures. Probiotic microorganisms help maintain intestinal balance and reduce the effects of gastrointestinal disorders such as antibiotic-associated diarrhea, inflammatory bowel disease, childhood diarrhea, traveler's diarrhea, lactose intolerance, irritable bowel syndrome, and intestinal diseases caused by *Clostridium difficile* (Quinto et al., 2014). The role of LAB in the production of tempoyak is crucial, as it directly impacts the product's quality. Therefore, identifying and exploring LAB is essential to ensure the production of high-quality tempoyak. The importance of probiotic foods, which currently dominate 60-70% of the functional food market. However, there is a need for deeper understanding of the specific microorganisms, particularly Lactic Acid Bacteria (LAB), in traditional fermented foods like tempoyak. While tempoyak is a nutritious probiotic food containing various nutrients, the precise characterization of its microbial composition remains incomplete. The novelty of this research lies in its comprehensive polyphasic approach, which combines morphological, physiological, biochemical, and molecular characteristics to systematically characterize LAB species in tempoyak from South Sumatra. The primary objectives of the study is to identify LAB species present in tempoyak from South Sumatra use a polyphasic approach to comprehensively characterize these bacterial isolates.

2. METHOD

This type of research uses a mixed method, which combines qualitative and quantitative methods. Qualitative methods were used to isolate, purify, and identify the morphology and biochemistry characters of LAB. Quantitative methods are used to identify LAB molecularly. The equipment used in this study included test tubes, test tube racks, petri dishes, micropipettes, microtips, Laminar Air Flow, tweezers, paper discs, incubator, centrifuge, waterbath, magnetic stirrer, spatula, drip pipette, burette, static Polymerase Chain Reaction (PCR) machine, analytical balance, microscope, glass slide, inoculation loop, falcon tubes, microtubes, electrophoresis apparatus, gel documentaion system, perforator, vernier calliper, pH meter, glass jar and spectrophotometer. The materials used include tempoyak from the Musi Rawas Regency area of South Sumatra Province, durian fruit, distilled water, De Man - Rogosa - Sharpe (MRS) agar (Merck), MRS broth (Himedia), Kovac's reagent, 3% H_2O_2, Gram stain, immersion oil, 70% alcohol, spritus, methyl red reagent, Barrit A reagent (alpha naphtol), Barrit B reagent (40% KOH), MR-VP medium (Himedia), SIM (Sulfur Indole Motility) medium (Merck), Simon Citrat medium (Merck), urea agar medium (Himedia), skim milk agar (Himedia), glucose (Merck), sucrose (Merck), lactose (Merck), mannitol (Merck), bactopepton (Oxoid), NaCl (Merck), bromothymol blue, 10% trichloroacetate, NaOH, HCl, KCl, Na_2HPO_4, KH_2PO_4, phenolphthalein indicator, oxalic acid, Bovine Serum Albumine (BSA), Bradford reagent, erythromycin, tetracycline, chloramphenicol, *Staphylococcus aureus, Salmonella typhimurium, Escherichia coli*, chicken ileum, Zymo Research kit, EF-Taq PCR kit (SolGent, Korea) and universal primers 27F (5' AGA GTT TGA TCM TGG CTC AG 3') and 1492R (5' TAC GGY TAC CTT GTT ACG ACT T 3').

LAB isolates were obtained from tempoyak samples from South Sumatra. LAB isolation was perfomed using MRS agar media (Merck) incubated at 37°C for 72 hours. The steps included diluting 1 gram of tempoyak in 9 ml of distilled water. Serial dilutions were made from 10^{-1} to 10^{-6}. The last three dilution (10^{-4}, 10^{-5}, 10^{-6}) were plated on sterile MRS agar media, which had been suplemented with 1 drop of nystatin. The samples were inoculated using the pour plate method, and the procedure was performed in duplicate. One milliliter of each dilution (10^{-4}, 10^{-5}, 10^{-6}) was mixed with MRS agar. The samples and media were then incubated at 37°C for 72 hours. Colonies that grew on the MRS agar and exhibited different morphological characteristics were purified using the streak plate method. Pure colonies were identified by the presence of single colonies along the streak lines. The pure colonies were propagated, and stock cultures were prepared. The stock cultures will be used for further testing including LAB identification, screening for the best probiotic candidate and tempoyak quality testing (Nizori et al., 2019). Pure isolates of LAB were identified using a polyphasic approach by analyzing morphological, biochemical, physiological and molecular characteristics. The morphological characteristics observed included colony morphology (shape, edge, elevation, size, appearance, optical properties, color) and cell morphology (shape and Gram properties). The physiological and biochemical characteristics of LAB were examined through test such as oxygen demand, motility, indole, catalase, MR-VP, citrate, urea, and sugar fermentation. These characteristics were compared with those in *Bergey's Manual of Determinative Bacteriology* (Khushboo, Arun Karnwal, 2020; Soleha & Retnaningrum, 2020). The morphology of LAB

colonies was observed by streaking purified isolates on MRS agar media in quadrants and incubating them for 72 hours at 37°C. The observed morphological traits included colony shape, edge, elevation, size, appearance, optical properties and color (Andhikawati & Permana, 2022). Cell shape and gram characteristics were observed using the Gram staining method. The glass slide was cleaned using 70% alcohol and air-dried. One drop of sterile distilled water was placed on the slide, and a loopful of the LAB isolate was spread across it. The bacterial preparation was stained with Crystal violet for 60 seconds, rinsed and dried. It was then treated with iodine for 60 seconds, rinsed and dried again. The preparations was decolorized with 95% alcohol for 30 seconds, rinsed, and stained with safranin for 60 seconds before being rinsed and dried. The cells were observed under a microscope at 100x magnification with immersion oil to determined their shape (cocci, rods and spirals). Gram-negative cells appeared red, while Gram-positive cells appeared purple (Ferdous et al., 2020) Oxygen demand in LAB was determined by examining their growth in MRS broth. A mixture of 5 grams of MRS broth and 90 ml of distilled water was homogenized and sterilized in an autoclave for 15 minutes (121°C, 15 psi). One loopful of LAB was inoculated into the sterile MRS broth and incubated at 37°C for 72 hours). After incubation, the bacterial growth pattern was observed. Obligate aerobic bacteria grew on the media surface, Facultative anaerobes grew throughout the media with concentrated growth on the surface, microaerophiles grew just below the surface, and obligate anaerobes grew at the bottom (Sionek et al., 2024).

The motility test was performed using SIM (Sulfur Indol Motility) and MRS agar media. Each LAB isolate was inoculated into the agar using a straight needle and incubated for 72 hours at 37°C. Motile LAB isolates showed spreading from the inoculation point (Rahayu & Setiadi, 2023). The Indole test assessed the ability of LAB isolates to degrade tryptophan. LAB isolates were inoculated into SIM agar and incubat at 72 hours at 37°C. After incubation, 10 drops of Kovac's reagent were added. A red layer on the media surface indicated a positive result (da Silva et al., 2020). The catalase test used 3% H_2O_2 reagent. The cleaned glass slide was covered with 2 drops of 3% H_2O_2, and one loopful of LAB isolate was added. The presence of bubbles indicated a positive test for catalase activity, reflecting the breakdown of hydrogen peroxide into oxygen and water (Rahayu & Setiadi, 2023). The MR-VP test consisted of two parts: the MR test, using methyl red, and the VP test, using Barrit A (α-naphtol) and Barrit B (40% KOH) reagents. LAB isolate were inoculated into 5 ml of MR-VP broth, incubated at 37°C for 72 hours, and tested in duplicate. In the MR test, a red color indicated positive result. For the VP test, 0.6 ml of Barrit A and 0.2 ml of Barrit B were added to the broth, and a red color signified a positive result (Lingga et al., 2023). The citrate test was performed using Simmons Citrate media. Simmons Citrate slant media was made by dissolving 1.2 grams of Simmons Citrate media in 50 ml of distilled water. LAB isolates were inoculated onto Simmons Citrate slants, and incubated at 37°C for 72 hours. A color change from green to blue indicated a positive result (Goa et al., 2022). The urea test used urea agar, prepared by dissolving 1.3 grams of urea agar into 50 ml of distilled water. Distribute into 10 test tubes, each as much as 5 ml. The test tube was tilted to form a sloping agar. LAB isolates were inoculated on the slanted agar and incubated at 37°C for 72 hours. A positive result was indicated by a color change from yellow to red, showing the decomposition of urea to ammonia by the enzyme urease enzyme) (Dahlén et al., 2018). The sugar fermentation test used media containing glucose, lactose, mannitol and sucrose. The medium consisted of bactopeptone (10 g/L), NaCl (5 g/L), 10 g/L sugar (glucose, sucrose, lactose, mannitol) and bromothymol blue. LAB isolate were inoculated into the sugar media and incubated at 37°C for 72 hours. A positive result was indicated by the a yellow color, reflecting acid production from fermentation (Hadi et al., 2019; Zamanpour et al., 2023). Molecular identification was performed by sequencing the 16S rRNA gene. Genomic DNA was isolated and the was amplified using universal primers 27F (5' AGA GTT TGA TCM TGG CTC AG 3') and 1492R (5' TAC GGY TAC CTT GTT ACG ACT T 3'). The PCR reaction mixture consisted of 20 ng of genomic DNA and 30 µl of EF-Taq PCR kit (SolGent, Korea), following the protocol: pre-denaturation at 95°C for 2 minutes, denaturation for (35 cycles at 95°C for 1 minute, annealing at 55°C, for 1 minute), elongation at 72°C for 1 minute), and final elongation at 72°C for 10 minutes. PCR products were purified using multiscreen filter plate (Millipore Corp., Bedford, MA, USA). PCR products were then sequenced (Soleha & Retnaningrum, 2020). The Ab1-format DNA sequence were edited using GeneStudio Pro 2.2 and converted to FASTA-format using ClustalX 2.1, and compared with the DNA database using BLAST. Phylogenetic relationship were reconstructed using the Maximum likelihood method in MEGA X with 1000 a bootstrap replicates (Soleha & Retnaningrum, 2020).

3. RESULT AND DISCUSSION

Result

The isolation process resulted in 8 LAB isolates identified at dilution levels of 10^{-4}, 10^{-5} and 10^{-6}. These eight isolates were coded as TP01, TP02, TP03, TP04, TP05, TP06, TP07 and TP08. Isolate TP01

was obtained from the 10^{-6} dilution series in the second replicate Petri dish, while isolates TP02, TP03, TP04 and TP05 were obtained from the 10^{-5} dilution series in the first replicate Petri dish. In the 10^{-4} dilution series of the first replicate Petri dish, isolates TP06 and TP07 were found, and isolate TP08 was observed in the second replicate Petri dish from the 10^{-4} dilution series. The purification process resulted in three LAB isolates that were able to grow and thrive on MRS agar media (Figure 1). These purified LAB isolates were TP02, TP07 and TP08.

Figure 1. Purification of LAB by Streak Plate Method

The identification of TP02, TP07 and TP08 isolates was performed based on colony morphological, cell morphology, physiological and biochemical characteristics, and molecular analysis on 16S rRNA gene fragments. The colony morphological characteristics observed from the pure colonies of TP02, TP07 and TP08 included colony shape, edge, elevation, size, appearance, optical properties and color. Isolate TP02 exhibited a circular shape, curled edges, convex elevation, medium size, shiny appearance, opaque optical properties and cream color. Isolate TP07 had cream-colored bacterial colonies with, opaque optical properties, a shiny appearance, medium size, pulvinate elevation height, curled edges, and a circular shape. Each of the three isolates showed distinct colony morphological characteristics (Table 1). The cell morphology of LAB was determined through Gram staining. Microscopic observation of isolates TP02, TP07 and TP08 showed that the cells of all three LAB isolates were strongly stained purple and rod-shaped (Table 2).

Table 1. Morphological Characterization of LAB Colonies

Isolate	Colony Morphology						
	CS	E	H	S	A	OP	CC
TP02	circular	curled	convex	Medium	Shiny	opaque	cream
TP07	circular	curled	pulvinate	Medium	Shiny	opaque	cream
TP08	irregular	undulate	convex	Medium	shiny	opaque	cream

Description: CS (Colony Shape), E (Edge), H (Height), S (Size), A (Appearance), OP (Optical properties) and CC (Colony Color).

Table 2. Characterization of LAB cell Morphology

Isolate	Cell Morphology		
	Cell Shape	Cell Color	Gram properties
TP02	Rod	Purple	Positive
TP07	Rod	Purple	Positive
TP08	Rod	Purple	Positive

The physiological and biochemical characteristics of LAB were observed through tests for oxygen demand, motility, indole production, catalase activity, the MR-VP test, citrate utilization, urea hydrolysis, and sugar fermentation. These tests are crucial for distinguishing bacterial species. Oxygen demand, motility, indole, catalase, VP, citrate and urea tests for isolates TP02, TP07 and TP08 yielded negative results (Table 3). Positive results were observed in the MR test and sugar fermentation test, with the exception of the mannitol sugar test, where TP02 and TP08 showed negative results. Variations in LAB's physiological and biochemical tests results influences species identification (Table 3).

Table 3. LAB Physiology and Biochemical Tests

Isolate	Parameters										
	O_2	Mo	Ind	Kat	MR	VP	Ct	U	Sugar test			
									G	S	L	M
TP02	Anaerobic	-	-	-	+	-	-	-	+	+	+	-
TP07	Anaerobic	-	-	-	+	-	-	-	+	+	+	+
TP08	Facultative anaerobes	-	-	-	+	-	-	-	+	+	+	-

Description: Mo (Motility), Ind (Indol), Kat (Catalase), Ct (Citrate), U (Urea), G (Glucose), S (Sucrose), L (Lactose) and M (Mannitol).

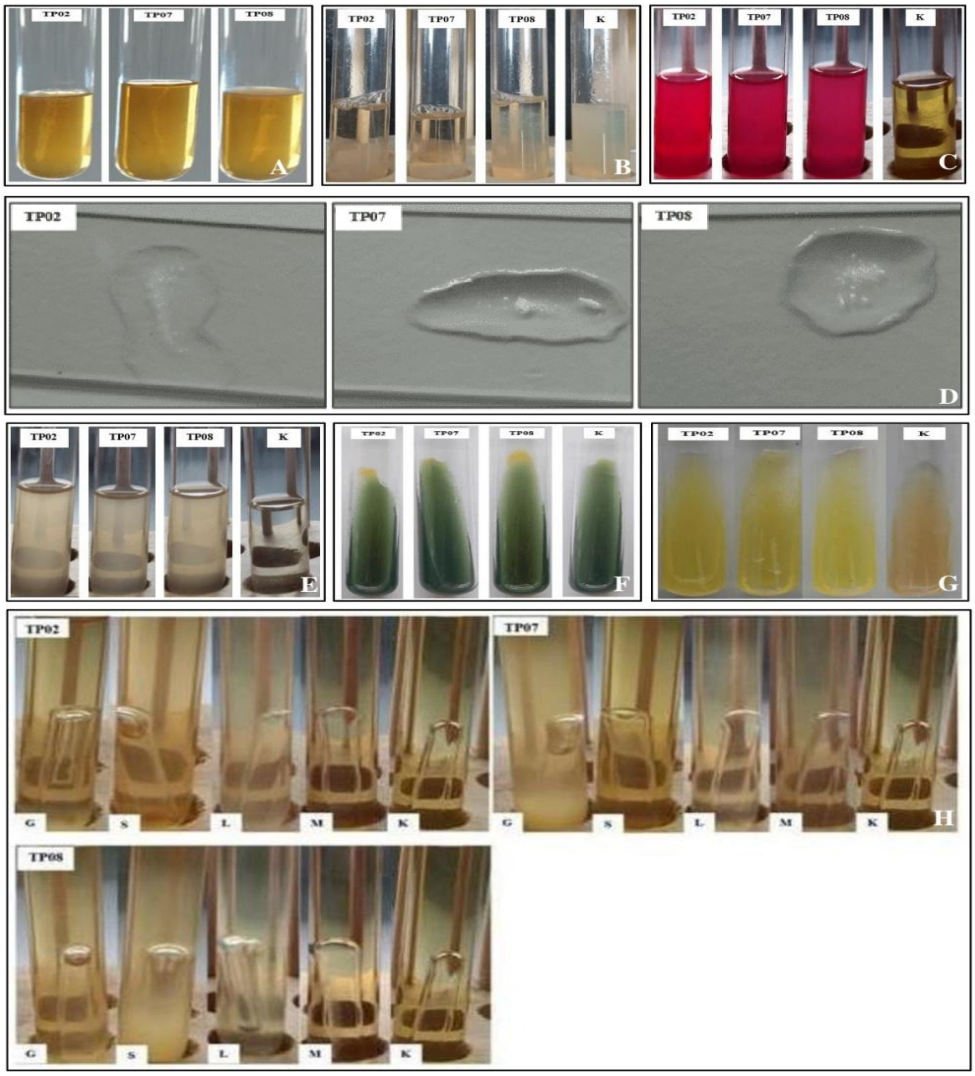

Figure 2. LAB Physiology and Biochemical Test on Isolates TP02, TP07 and TP08. Motility test (A); Indole Test (B); MR Test (C); Catalase Test (D); VP Test (E); Citrate Test (F); Urease Test (G); Sugar Test (H); Control: Test without Lactic Acid Bacteria Isolate (K); Glucose (G); Sucrose (S), Lactose (L), Mannitol (M)

The results of 16S rRNA gene sequencing for isolates TP02, TP07 and TP08 using universal primers 27F (5' AGA GTT TGA TCM TGG CTC AG 3') and 1492R (5' TAC GGY TAC CTT GTT ACG ACT T 3') produced DNA strand approximately 1500 bp in length. The quality of the sequencing results was validated using the Sequence Scanner application. Sequencing quality was assessed based on the Quality Value (QV) of pure & mixed bases and the peak intensity of nitrogen bases. The pure base QV measure the accuracy with which nitrogen bases are identified as adenine (A), guanine (G), thymine (T), or cytosine (C). The mixed base QV is used to assess the sequencing quality when these bases can not be clearly distinguished. Based on QV values, the 16S rRNA gene sequencing results for isolates TP02, TP07 and TP08 showed high quality, with a QV value ≥ 20. In addition to using the QV value, the quality of the 16S

rRNA gene sequence can also be assessed by the height and clarity of nitrogen base peak. The gene sequence has an optimal peak (Figure 3).

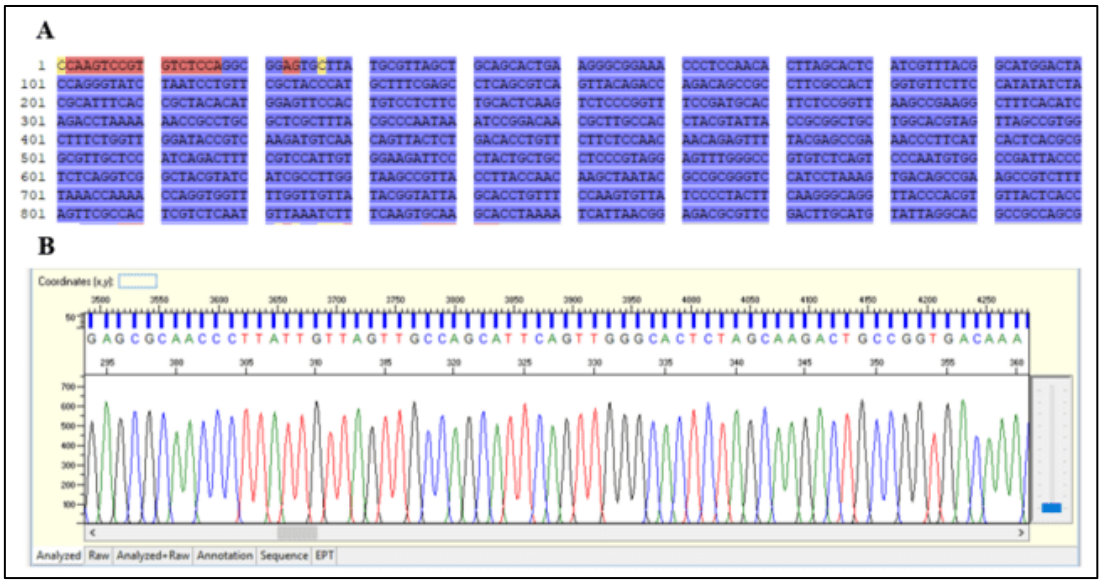

Figure 3. The Quality of 16S rRNA Gene Sequencing Results. A (Quality Value) and B (Nitrogen Base Peak)

Once the 16S rRNA gene sequence has been confirmed to be of good quality, it is BLASTed using the tool at https://blast.ncbi.nlm.nih.gov/Blast.cgi. This step compares the 16S rRNA gene sequence from the research with sequences in GenBank. The BLAST results revealed that the 16S rRNA gene sequence of isolate TP02 closely matched the genus *Lactobacillus,* while the sequence of isolate TP07 and TP08 matched the genus *Lentilactobacillus* (Table 4). These molecular identification result were consistent with the findings from phenotypic characterization.

Table 4. BLASTn Results of 16S rRNA Gene Sequences for Isolates TP02, TP07 and TP08

Isolate	Query Cover (%)	Identity (%)	Closest Species	*Accession*
TP02	99	99,93	*Lactobacillus buchneri* strain DG1	LC094428.1
	99	99,93	*Lactobacillus* sp,YIT 11469	AB462914.1
	99	99,88	*Lactobacillus buchneri* strain TB-H34	AB425940.1
	99	99,87	*Lactobacillus dextrinicus* strain SN5	LN870301.1
	99	99,87	*Lactobacillus buchneri* strain FD2	JN188387.1
TP07	80	96,22	*Lentilactobacilus kefiri* strain NBRC 15888	NR_113336.1
	80	96,22	*Lentilactobacillus parabuchneri* strain LMG 11457	NR_114962.1
	80	96,22	*Lentilactobacillus parabuchneri* strain JCM 12493	NR_041293.1
	80	96,22	*Lentilactobacillus buchneri* strain JCM 1115	NR_041293.1
	80	96,22	*Lentilactobacillus parakefiri* strain JCM 8573	NR_112757.1
	80	96,08	*Lentilactobacilus sunkii* strain YIT 11161	NR_041656.1
	80	95,95	*Lentilactobacilus parakefiri* strainNBRC 15890	NR_113819.1
	80	96,08	*Lentilactobacillus parabuchneri* strain DSM 5707	NR_112755.1
	80	96,08	*Lentilactobacillus kisonensis* strain YIT 11168	NR_041658.1
	80	95,93	*Lentilactobacillus otakiensis* strain YIT 11163	NR_041657.1
TP08	56	99,60	*Lentilactobacillus buchneri* strain JCM 1115	NR_041293.1
	56	98,94	*Lentilactobacillus sunkii* strain YIT 11161	NR_041656.1
	56	98,81	*Lentilactobacillus otakiensis* strain YIT 11163	NR_041657.1
	56	98,87	*Lentilactobacillus parakefiri* strain NBRC 15890	NR_113819.1
	56	99,00	*Lentilactobacillus parakefiri* strain JCM 8573	NR_112757.1
	56	98,61	*Lentilactobacillus parabuchneri* strain JCM 12493	NR_041294.1
	56	98,48	*Lentilactobacillus parabuchneri* strain LMG 11457	NR_114962.1
	56	98,35	*Lentilactobacillus rapi* strain YIT 11204	NR_041659.1
	56	98,54	*Lentilactobacillus kefiri* strain NBRC 15888	NR_113336.1
	56	98,47	*Lentilactobacillus parabuchneri* strain DSM 5707	NR_112755.1

The 16S rRNA gene sequences of the species listed in Table 4 were used to create a phylogenetic tree. The results of the phylogenetic tree construction showed that TP02 was closely related to *Lactobacillus* sp, YIT 11469, TP07 was closely related to *Lentilactobacillus kefiri* strain NBRC 15888 and TP08 was closely related to *Lentilactobacillus buchneri* strain JCM 1115 (Figure 4).

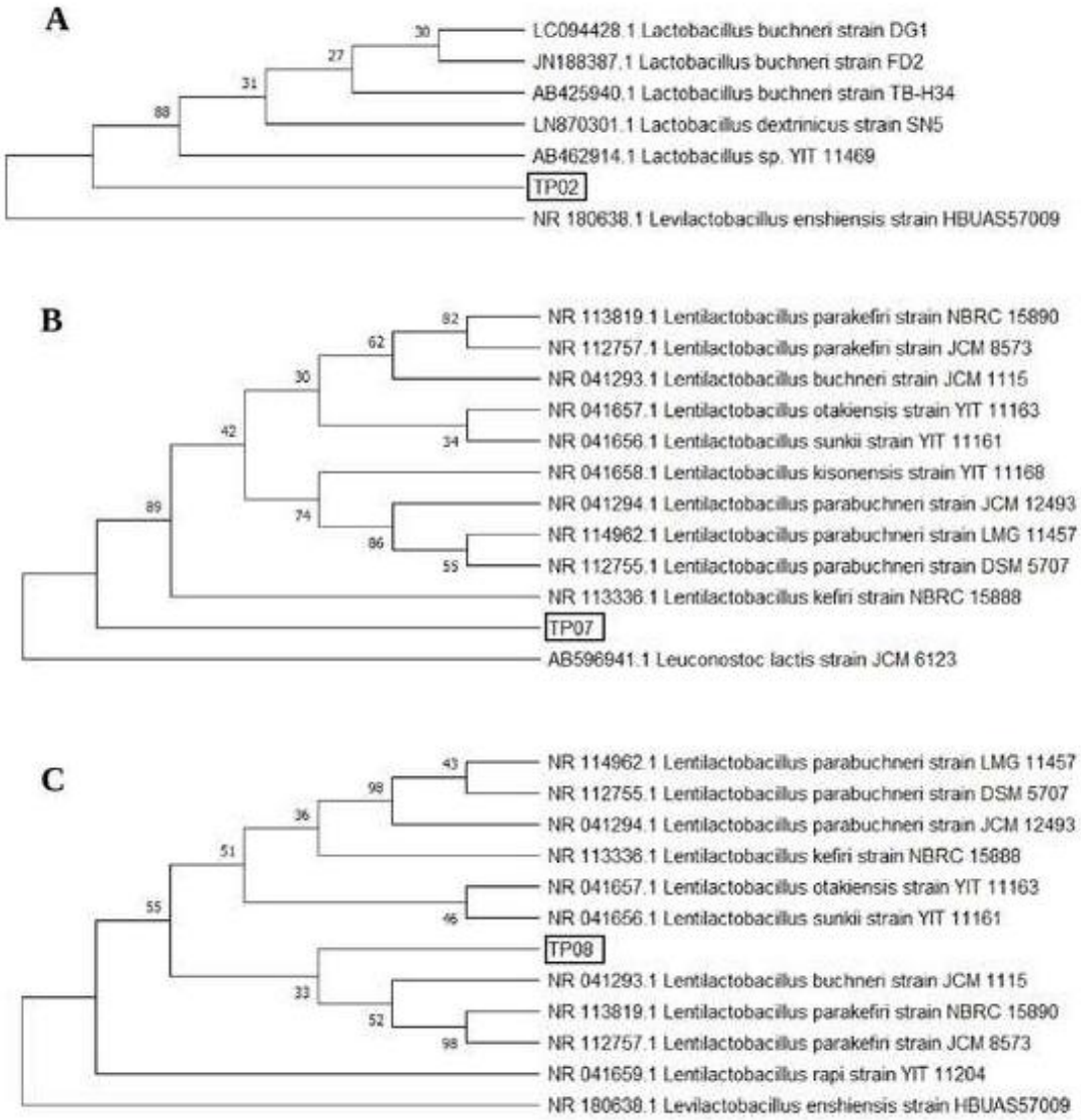

Figure 4. Phylogenetic Tree of Lactic Acid Bacteria Isolates. A: TP02, B: TP07 and C; TP08

Discussion

The isolation of Lactic Acid Bacteria (LAB) from tempoyak samples collected in South Sumatra was performed using the pour plate method. The pour plate method is effective for isolating both aerobic and anaerobic bacteria simultaneously, as Aerobic bacteria grow on the surface of the agar, while anaerobic bacteria grow within the agar layers (Suradeep & Prathapkumar, 2021). This method is widely employed across various analyses due to its simplicity, cost-effectiveness and high accuracy. It is commonly used in the microbiological analysis of food, environmental pollution assessments, as well as in the cosmetics, pharmaceutical and other industries (Erkmen, 2021). Selection of isolates was based on distinct morphological characteristics of LAB colonies on the MRS agar surface. Colony morphological characteristics can be used for simple identification of bacteria (Obioha et al., 2021). Therefore, morphological characters can be a useful tool for the preliminary identification of bacterial species or strains. The purity of the 8 isolates from isolation process was confirmed using the streak plate method. This method is designed to isolate pure colonies from mixed populations by mechanical separation. Pure colonies are characterized by distinct, separate single colonies growing on the agar surface. Single colony consist of millions of bacterial cells growing together as a group (Huligere et al., 2023). Isolates TP01,

TP03, TP04, TP05 and TP06 did not grow during the purification process. The failure of TP01, TP03, TP04, TP05 and TP06 to grow in purification step could be attributed to various physical and chemical factors. Environmental factors such as pH, temperature, osmotic pressure, oxygen levels and the presence of heavy metal, as well as micronutrients imbalances (e.g. , insufficient or excessive minerals and vitamins), can inhibit bacterial growth (Maynard & Weinkove, 2020). The purification process obtained three pure isolates coded as TP02, TP07 and TP08. These three isolates were identified based on colony morphology, cell morphology, physiology, biochemistry and molecular characteristics. The characteristics of bacterial colonies and gram properties are the keys to identifying bacteria. Colony morphology and gram properties can assist in identity bacteria, as different species or strains can exhibits varying morphological trait (Ferdous et al., 2020). The Gram stain technique aims to classify bacteria based on cell shape and gram reaction. Gram characteristics are determined by the ability of bacterial cell walls to retain color reagents. Gram-negative bacteria lose the purple color from crystal violet when treated with an alcohol solution, and then absorb of safranin, resulting in a red color. In contrast, Gram-positive bacteria retain the purple color from crystal violet, producing a purple hue (Sianipar et al., 2020). The gram-positive bacterial group can retain crystal violet because their the bacterial cell wall contains simpler lipids compared to gram-negative bacteria. As a result, the cell walls of gram-positive bacteria are more easily degraded when treated with alcohol. The degraded cell wall causes the pore size to shrink, reducing cell permeability, and making it difficult for to be removed. Consequently, the violet stain remains bound to the cell. In contrast, gram-negative bacteria lose the crystal violet during the alcohol rinsing process, resulting in a red appearance after the safranin dye is absorbed at the end of the staining process. Gram-negative bacteria contain a higher lipid content than gram-positive bacteria (Yanti et al., 2022)

Gram staining shows that isolates TP02, TP07 and TP08 are a group of gram-positive rod-shaped bacteria (Table 2). This confirms that the isolates belong to the gram-positive bacterial group. These findings are consistent with the study, which also identified lactic acid bacteria as part of the Gram-positive bacteria group (Al-Mohammadi et al., 2021). The gram characteristics and cell shape of isolates TP02, TP07 and TP08 are consistent with members of the LAB genera *Lactobacillus*, *Lentilactobacillus* and *Bifidobacterium*. These genera are gram positive, rod shaped (bacillus), and may appear singly or in chains (monobacilli, cocobacilli, diplobacilli and streptobacilli). LAB are gram-positive bacteria that are either round or rod-shaped (monobacilli, cocobacilli, diplobacilli and streptobacilli), non spore forming catalase negative, and capable of converting carbohydrates into lactic acid (Ramadhanti et al., 2021). Gram-positive bacteria retain crystal violet dye due to the presence of teichoic acid in their peptidoglycan layer.
The tests used to determine the physiological and biochemical characteristics of three bacterial isolates were oxygen demand, motility, indole, catalase, MR-VP, citrate, urea, and sugar fermentation tests. The oxygen demand of LAB isolates TP02, TP07 and TP08 were assessed by observing their growth patterns in MRS broth media. isolates TP02 and TP07 demonstrated anaerobic growth, as they grew at the bottom of the MRS broth a region devoid of oxygen (Table 3). Anaerobic bacteria grow and thrive in environments without oxygen, obtaining energy through fermentation, with–organic compounds serving as the final electron acceptors (Buckel, 2021).

In contrast, isolate TP08 exhibited growth that extended from just below the surface to the bottom of the MRS broth indicating that it is facultative anaerobic (Table 3). Facultative anaerobes can grow in both oxygenated and oxygen-poor environments. In oxygen-rich condition, facultative anaerobes generate ATP through respiration, while in oxygen-poor conditions, they rely on fermentation for ATP production. is obtained through the fermentation process. Facultative anaerobic bacteria are highly adaptable, utilizing oxygen as the final electron acceptor during in the respiration, or organic compounds during fermentation when oxygen is absent (André et al., 2021).

The motility of LAB isolates was observed using the stab method upright MRS agar media. Motility refers to an organism's ability to move, which is typically facilitated by the flagellum, a thread-like structur embedded in the bacterial cell membrane and wall, acting as a locomotor. The motility test aims to assess the ability of microbes to move, and motility is a crucial parameter in taxonomy, particularly in bacterial classification (Palma et al., 2022). The motility ability of the three LAB isolates from *tempoyak* in South Sumatra showed that all isolates were non-motile. No blurring or root-like spreading was observed from the puncture line in the MRS agar media (Figure 2). Root-like growth around the inoculation site typically indicate bacteria movement, but in this case, the absence of such growth suggests that the bacteria lack flagella (Zheng et al., 2021). Lactic acid bacteria isolated from kimchi are also non-motile (Ismail et al., 2023). Non-motile bacteria exhibit limited growth, only occuring around the puncture area created by the inoculating needle. This motility test result is consistent with the general characteristic of LAB, which are typically non-motile (Rahayu & Setiadi, 2023). The ability of LAB isolates to produce the enzyme tryptophanase was assessed using the indole test. Triptopanase is an enzyme capable of hydrolyze the amino acid tryptophan. As one of the essential amino acids, tryptophan is vital for supporting the growth

and survival of bacteria. Tryptophan is commonly found in protein sources and is readily utilized by microorganisms as an energy source (Barik, 2020). The indole test for isolates TP02, TP07 and TP08 showed negative results, indicating that these isolates do not produce the enzyme triptopanase. Negative results are characterized by the absence of a red ring and the formation of a yellow layer on the surface of SIM media (Figure 2). The red ring forms due to the reaction between indole in SIM media and p-dimethylaminobenzaldehyde in Kovac's reagent. The yellow layer indicates that the butanol component of kovac's reagent did not bind to indole in SIM media, preventing a reaction with p-dimethylaminobenzaldehyde. The reaction between indole and p-dimethylaminobenzaldehyde form a red complex that would appear on the surface of SIM media (Walusansa et al., 2022).

The MR test is used to determine a bacterium's ability to carry out mixed acid fermentation. The MR test results show that all three isolates tested positive. Positive results are characterized by a color change in the media from yellow to red/reddish after the addition of Methyl Red indicator (Figure 2). The red color in MR test indicates a decrease in the pH of the media due to the production of large amounts of acid from glucose fermentation (Khushboo & Arun Karnwal, 2020). The MR test on *E. coli* isolates also showed positive results, as indicated by the red color change in the media. This occurs because organisms that performs mixed acid fermentation produce sufficient acid to lower the pH (Asha et al., 2024). The catalase test aims to determine the activity of the catalase enzyme in hydrolyzing hydrogen peroxide (H_2O_2) into water (H_2O) and oxygen (O_2). A positive result is indicated by the formation of air bubbles while a negative results shows no bubbles (Sianipar et al., 2020). The catalase test for isolates TP02, TP07 and TP08 showed negative results when reacting with H_2O2 (Figure 2). These findings suggest that the three isolates are homofermentative. This is consistent with the research, where a negative catalase test was indicated by the absence of bubbles after the addition of H_2O_2 (Khushboo, Arun Karnwal, 2020; Pei et al., 2020). The absence of bubbles in the catalase test suggested that the three LAB isolates do not produce the enzyme catalase, which is required to break down hydrogen peroxide (H_2O_2) into water (H_2O) and oxygen (O2). Lactic acid bacteria are unable to produce the catalase enzyme, which break down H_2O_2, noted that LAB does not produce catalase, as it only requires minimal oxygen to survive (Bryukhanov et al., 2022; Li et al., 2022). LAB are facultative anaerobic bacteria, typically found in diverse habitats such as the digestive tract of animals and humans, canned foods, dairy products, fermented products, tropical fruits and vegetables (Coleman et al., 2021). LAB has been widely used as a food preservative, fermentation culture, and probiotic due to its anti-microbial properties and ability to prevent food spoilage.

VP (Voges Preskauer) test is used to determine the ability of bacteria to produce neutral end products from glucose fermentation through the butanediol pathway. According to Windy & Dewi (2023), the Voges Proskauer test is conducted to assess the ability of certain organisms to form non-acidic or neutral end products from the organic acids produced during glucose metabolism. The results of the research on the three bacterial showed negative results (Figure 2), indicated by the absence of color changes in the media after adding the indicator (Manalu et al., 2020). Citrate test assesses the ability of bacteria to use citrate as the only source of carbon and energy (Medaando et al., 2024). The citrate test was conducted by inoculating bacterial isolates onto Simon citrate Agar media, followed by incubation at 37ºC for 48 hours. The results of the citrate test indicated that all isolates were negative (Figure 2). In Simon Citrate media, negative results were obtained, as indicated by the media not changing to blue. This suggests that the bacteria do not utilized citrate as a carbon sources. The citrate test for all isolates was negative, with no color change observed in the tested media. Citrate is known as a weak organic acid found in leaves and Citrus plants. These findings are consistent with the results, which also indicated that LAB from all isolates were unable to use citrate as a source of carbon and energy, as evidenced by the lack of color change in the test media (Khushboo et al., 2023). The urease test aims to differentiate organisms based on the ability to hydrolyze urea using the enzyme urease. Isolates TP02, TP07, and TP08 did not hydrolyze urea at all, indicated by the media remaining yellow (Figure 2). If the urea agar medium turns pink within 24 hours, it indicates rapid urea hydrolisis and strong urease production (Mekonnen et al., 2021). If the medium is partially pink within 24 hours, it suggest slow urea hidroysis and weak urea production. If the medium remains orange or yellow within 24 hours but turns partly pink within 6 days, this indicates slow urea hydrolisis and weak urease production. If the medium stay orange or yellow without changing color by the 6th day, it means the organism does not hydrolyze urea and does not produce urease.

The ability of isolates TP02, TP07 and TP08 to ferment carbohydrates was observed using a sugar fermentation test. The sugar sources used were glucose, sucrose, lactose and mannitol, representing monosaccharides, disaccharides and sugar alcohols. Glucose serves as the representative substrate for the monosaccharide group, sucrose and lactose for the disaccharide group, and mannitol for the sugar alcohol group. Glucose is particularly important in this test as it is a simple carbohydrate that is readily utilized

first in the fermentation process (Gunkova et al., 2021). Sugar sources are essential for all heterotrophic organisms, as they are used as substrates in metabolic processes. The metabolism of isolates TP02, TP07, and TP08 occurs anaerobically, known as fermentation. Fermentation is a biochemical reaction that converts glucose into organic acids, carbon dioxide and alcohol. This process involves microorganisms and uses organic molecules as the final electron acceptor (Darmadi et al., 2020). The growth of TP02, TP07, and TP08 on mannitol media did not result in gas production in Durham tubes, and the media was less turbid when compared to the control (Figure 2). These results indicate that the three LAB isolates have varying abilities to ferment different sugars. The variation in LAB's ability to ferment sugar is an important factor in the identification of bacterial species. Different bacterial species have distinct abilities to ferment specific carbohydrates, while some bacteria can ferment the same carbohydrates, others cannot (Wang et al., 2021).

The sugar fermentation test showed that not all lactic acid bacteria (LAB) isolates were capable of utilizing the various sugar provided in the test media (Table 3). Table 3 shows that LAB isolates produced gas in glucose (G), sucrose (S) and lactose (L) media, making the media much more turbid compared to the control. This is further supported by the MR test, which demonstrates that LAB isolate are capable of producing organic acids during the fermentation process. The results of the sugar test are consistent with the research, which reported that LAB isolates could ferment glucose, lactose, maltose, mannitol, and sorbitol (Adikari et al., 2021; Medaando et al., 2024). The Universal primers, 27F (5' AGA GTT TGA TCM TGG CTC AG 3') and 1492R (5' TAC GGY TAC CTT GTT ACG ACT T 3'), are designed to identified bacteria based on their 16S rRNA gene sequence. The primers produced DNA bands and sequences of 1500 bp, consistent with findings by (Suphandi et al., 2023). Sequencing quality was assessed based on the Quality Value (QV) of pure & mixed bases and the peak intensity of nitrogen bases. Based on QV values, the 16S rRNA gene sequencing results for isolates TP02, TP07 and TP08 showed high quality, with a QV value ≥ 20. Nitrogenous bases with a QV value ≥ 20 have a minimal error rate of just 1% (Gaffar & Sumarlin, 2021). Nitrogenous bases with a QV value ≥ 20 are marked in blue, medium- quality bases are (QV 15-19) are marked in yellow, and low-quality bases (QV≤ 15) are marked in red. Based on the figure, the nitrogen bases from the sequencing results exhibit optimal peaks. The peak indicates the quality of the nitrogen base. An optimal peak suggests that the sequenced DNA is of high quality. A good peak is characterized by a symmetrical shape, where peaks are clearly separated and do not overlap (Al-Shuhaib & Hashim, 2023). In contrast, a less-than-optimal peak is asymmetrical and indicates lower nitrogen base quality. Such peaks can result from factors like excess samples, sample degradation, or contamination. Nitrogenous bases with a QV value ≥ 20 have a minimal error rate of just 1%. Nitrogenous bases with a QV value ≥ 20 are marked in blue, medium- quality bases are (QV 15-19) are marked in yellow, and low-quality bases (QV≤ 15) are marked in red (Gaffar & Sumarlin, 2021).

Isolate TP02 had a high similarity to *Lactobacillus buschneri* strain DG1 and *Lactobacillus* sp, YIT 11469, with similarity identity percentage of 99.93%. Isolate TP07 showed similarity to multiple of 16S rRNA gene sequence with species *Lentilactobacillus* species, including L. *kefiri* strain NBRC 15888, *L. parabuchneri* strain LMG 11457, *L. parabuchneri* strain JCM 12493, *L. buchneri* strain JCM 1115, *L. parakefiri* strain JCM 8573 and *L. sunkii* strain YIT 11161, with a similarity percentage of 96.22%. Isolate TP08 had very high similarity (99,60%) with *Lentilactobacillus buchneri* strain JCM 1115. This percentage suggests that TP02 and TP08 are closely related at the strain level, while isolate TP07 may represent a new species. A 16S rRNA gene sequence identity percentage greater than > 99.5% is required to identify isolates at the strain level, while 99% is the threshold for species-level identification (Lin et al., 2023). A Percentage identity between 97-99% is used genus-level identification, and a percentage below of 97% may indicate a potential new bacterial species.

The kinship relationships of isolates TP02, TP07 and TP08 were analyzed using a phylogenetic tree. The construction of the phylogenetic tree begins with the determination of outgroups, alignment of sample DNA sequences, and the application of the Maximum Likelihood method using Mega X. The outgroup chosen for creating the phylogenetic tree is *Levilactobacillus enshiensis*. This species was selected because it belongs to the same family as the genera *Lactobacillus* and *Lentilactobacillus*. The selection of an outgroup is a species outside the ingroup that is used to establish the root of the phylogenetic tree. Outgroups that have a genetic distance too close to the ingroup can reduce the quality of the resulting phylogenetic tree (Coleman et al., 2021). Isolate TP08 has a close evolutionary distance with the group that includes *Lentilactobacillus otakiensis* strain YIT 11163 and *Lentilactobacillus sunki* strain YIT 11161. This isolate is also closely related to *Lentilactobacillus buchneri* strain JCM 1115 and *the Lentilactobacillus parakefiri* group. Based on the phylogenetic tree analysis, it shows that isolate TP08 has a very close relationship with *Lentilactobacillus buchneri* strain JCM 1115. The construction of the phylogenetic tree shows that isolate TP02 has a large evolutionary distance and appears to be closer to *Lactobacillus* sp. strain YIT 11469 (bootstrap 88) compared to *Lactobacillus buchneri* and *Levilactobacillus*

enhiensis. Isolate TP07 is in a group consisting of *Lentilactobacillus parabuchneri* and *Lentilactobacillus kefiri*. The TP07 group is far from *Leuconostoc lactis* strain JCM 6123, indicating a more distant relationship between the genera *Lentilactobacillus* and *Leuconostoc*. TP07 is closely related to *Lentilactobacillus* species, especially *Lentilactobacillus kefiri* with a bootstrap value of 89. A high bootstrap value (above 70) indicates a strong confidence level in the phylogenetic tree. High bootstrap values can be used as an indicator of a phylogenetic tree's level of confidence. Phylogenetic trees with a high level of confidence are those with bootstrap above 70 (Rangkuti et al., 2022).

4. CONCLUSION

This study successfully isolated three strains of Lactic Acid Bacteria (LAB) coded as TP02, TP07 and TP08. Based on a polyphasic approach, TP02 was identified as *Lactobacillus* sp, YIT 11469, TP07 was identified as *Lentilactobacilus kefiri* strain NBRC 15888 and TP08 was identified as *Lentilactobacillus buchneri* strain JCM 1115. TP07 has similarities with *Lentilactobacilus kefiri* strain NBRC 15888 but still has the potential to be classified as a new species.

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