

Isolation and molecular characterization of probiotic from Sidamanik green tea (*Camellia sinensis*) fermentation

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Abstract. Fahmi A, Syukur S, Chaidir Z, Melia S. 2023. Isolation and molecular characterization of probiotic from Sidamanik green tea (*Camellia sinensis*) fermentation. *Biodiversitas* 24: 4277-4288. The bioavailability of green tea catechins is reduced in the gastrointestinal tract because of its instability. Probiotics are expected to increase their bioavailability in the gastrointestinal tract. There is no any information about Sidamanik fermented green tea, either probiotics, antimicrobial activity, or probiotic species that are beneficial to health. This research aims to isolate and test the characterization of probiotics from fermented green tea. Green tea from Sidamanik Sub-district was fermented with palm sugar and forest honey with 24, 48, and 72 h variation times. The fermented green tea was tested for morphological characterization, Gram staining, catalase test, and fermentation test. Then the resistance test to bile acids and salts and the antimicrobial activity test against three pathogenic bacteria *Escherichia coli* Q157:H7, *Staphylococcus aureus* ATCC25923, and *Listeria monocytogenes* CFSAN004330. Two strains were selected from this series of tests, which were identified using 16S rRNA gene sequencing from different green tea fermented at the same fermentation time. The results indicated a positive lactic acid bacteria (LAB) morphological test. The characteristics of LAB can be described as a single colony being seen with a round, smooth, yellowish-white color, and a clear zone was formed around the colony, positive Gram, negative catalase, and homofermentative. LAB strains 1.1., and 4.1 were chosen to test their antimicrobial activity because they had the best acid and salt resistance viability. Then the results of molecular identification of 16S rRNA gene found that the probiotics contained in LAB strain 1.1. was *Lactobacillus plantarum* Y-1 and in LAB strain 4.1 was *Lacticaseibacillus paracasei* HBUAS62903. This study aims to find a new product of fermented green tea that has benefits in digestive health because it contains naturally processed lactic acid bacteria, is cheaper, simpler, stable at room temperature, durable in storage, and easy to serve.

Keywords: 16S rRNA, antimicrobial activity, *Lactobacillus plantarum* Y-1, *Lacticaseibacillus paracasei* HBUAS62903, probiotic

INTRODUCTION

Tea leaves are produced by the tea (*Camellia sinensis*) plant, known for its various health advantages as a beverage. The quality of tea can be affected by this component. Tea from Sidamanik has a distinctive, fresh, and delicious taste influenced by geographical factors, climate, age of the leaves, types of picking and varieties, and clones. The Sidamanik tea plantation is located at Sidamanik Sub-district, Simalungun District, North Sumatra, Indonesia. Research on Sidamanik tea leaves is still rare and needs to be developed. Some of the research on the benefits of tea from Sidamanik had been done and also needed to be developed. The green tea ethanol extract has good antimicrobial activity against *Escherichia coli* (Fahmi et al. 2022a), *Pseudomonas aeruginosa* (Fahmi et al. 2022b), and *Propionibacterium acnes* (Fahmi et al. 2022c). Green tea generally contains catechins, namely (-) EGC (epigallocatechin), (-) GCG (gallicocatechin gallate), (-) -EGCG (epigallocatechin-3-gallate), (-) ECG (epicatechin-3 gallate). EGCG, the primary source of catechins in green tea, is beneficial for health and has been widely studied (Bae et al. 2020).

Green tea contains the most EGCG, so it is the focus of in vitro and in vivo studies. EGCG has powerful antioxidant activity but is unstable and has low bio-accessibility, when it enters the gastrointestinal tract. Therefore, it is necessary to increase bio-accessibility with probiotics in the form of lactic acid with fermentation. It makes food and beverages increase their value (Pedro 2020). Some of the fermented green tea research has been done too. Fermented green tea with *Eurotium cristatum* MF800948 improved the taste. The aroma of autumn green tea (Xiao et al. 2021), fermentation of tea infusion with *Saccharomyces boulardii* NCM I-745 and *Lactiplantibacillus plantarum* 299V increased and modulated tea flavor and developed a novel tea beverage with high counts of live probiotics (Wang et al. 2022), green tea supplementation with probiotics *Bifidobacteria* and *Lactobacillus* spp in 7-day showed the cecum microbiota and cecum/skin metabolism in mice (Jung et al. 2019), green tea yogurt with 2% green tea contain had better antimicrobial and antioxidant activity than black tea (Gulfem et al. 2018) and the new anti-obesity fermented product (combination of *Houttuynia cordate* leaves with green tea leaves using *Lactobacillus paracasei*) more effective than unfermented tea

(Wang et al. 2018). Fermented green tea is expected to improve gastrointestinal health with probiotics in green tea, which is rich in antioxidants.

Fermented green tea products still need to be developed because of their excellent benefits for digestive health. Several studies have been published on adding certain probiotic strains to green tea. There are also studies on adding fermented milk products to green tea and vice versa green tea as a supplement to fermented milk products. Sidamanik fermented green tea has never been published yet, either probiotics, antimicrobial activity, or probiotic species that are beneficial to health. This study aims to learn about that and find novelty in probiotics contained in fermented green tea from Sidamanik, North Sumatra. Fermented green tea could be a new product that benefits digestive health because it contains naturally processed lactic acid bacteria, is cheaper, simpler, stable at room temperature, durable in storage, and easy to serve.

MATERIALS AND METHODS

Sampling

This research used green tea from Sidamanik Sub-district, Simalungun District, North Sumatra, Indonesia. The map of Sidamanik tea plantation, 2°51'35.5"N 98°55'02.0"E (Figure 1).

Fermented green tea with forest honey

Weighed ± 20 g of green tea and then put it into a glass jar. Furthermore, weighed ± 4 mL of forest honey into a glass jar and 5 mL of warm distilled water (40°C), then stirred slowly with a spatula until the forest honey dissolved. Covered in a glass jar and placed in a closed room with variations of time 24, 48, and 72 h. It was then dried in a dehydrator at a low temperature (samples 1, 2, and 3) (Jin et al. 2021).

Fermented green tea with palm sugar

Weighed ± 20 g of green tea and then put it into a glass jar. Weighed ± 4 g of palm sugar into a glass jar and 5 mL

of warm distilled water (40°C) and then stirred slowly with a spatula until the palm sugar dissolved. Covered in a glass jar and placed in a closed room with variations of time 24, 48, and 72 h. It was then dried in a dehydrator at a low temperature (samples 4, 5, and 6) (Jin et al. 2021).

Isolation of lactic acid bacteria

An enrichment medium was prepared by dissolving 23.02 g MRS Broth (Merck) in 441 mL distilled water and 1% CaCO_3 , then heated while homogenizing with a hot plate stirrer at 100°C, allowed to drop to $\pm 55^\circ\text{C}$ and poured into an Erlenmeyer glass and autoclaved. MRS agar (Merck) media was prepared by dissolving 66.20 g of MRS agar in 1 L of distilled water, then heated while homogenizing with a hot plate stirrer at 100°C, allowed to drop to $\pm 55^\circ\text{C}$ and autoclaved, and then poured into each petri dish as much as ± 15 mL, allowed to solidify. Each sample was weighed and put into a test tube filled with 9 mL of MRS broth, closed and homogenized, then put into an anaerobic jar and incubated for 24 h at 37°C (as a 10^{-1} dilution). The results of 10^{-1} are taken 1 mL and put into a test tube containing 9 mL of MRS broth, closed and homogenized, then put into an anaerobic jar and incubated for 24 h at 37°C (as a 10^{-2} dilution), and so on until the dilution 10^{-6} . A hundred microliter was taken and planted from the dilutions of 10^{-6} and 10^{-7} as an inoculum using the spread method on a Petri plate containing MRS so that it was flattened using a hockey stick-like sterilized with alcohol and burned with Bunsen and aerated. Each Petri plate was marked, put into an anaerobic jar, and then incubated for 48 h at 37°C. The incubation Petri plate was taken and observed. The single colony was seen with a round shape, smooth, yellowish-white color, and a clear zone was formed around the colony, it indicated the characteristics of lactic acid bacteria. Furthermore, the single colony was purified by the streak method, namely taking a single colony using a sterile ose needle, streaking it on a petri dish containing MRS then, incubated for 48 hours at 37°C (Melia et al. 2019).

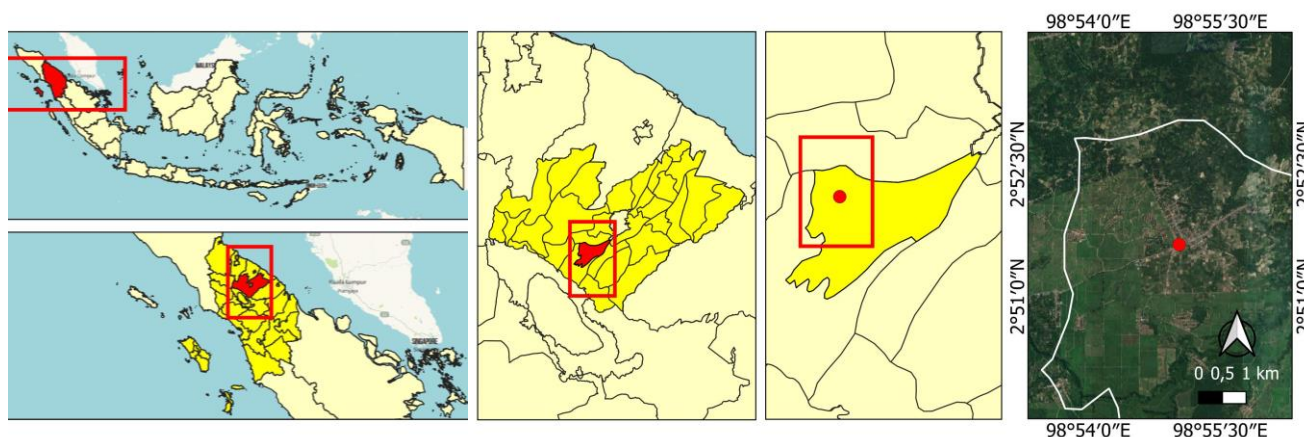


Figure 1. Map of green tea plantation in Sidamanik Sub-district, North Sumatra Province, Indonesia

Characteristics of LAB

LAB morphology

Morphological characteristics of LAB were observed macroscopically and microscopically. The observations were focused on colony shape, edge shape, and elevation.

Gram stain

The bacterial culture was taken, flattened on a slide, dripped with crystal violet, and waited for 60 seconds. After that, rinsed with distilled water, dripped with complex iodine solution, and waited 60 seconds. Then rinsed with running distilled water, washed with 96% alcohol, and rinsed with running distilled water. Dripped with safranin and then waited 30 sec and rinsed with distilled water) dried and examined under a microscope (1000×) using immersion oil. Gram-positive bacteria were shown in blue to purple and gram-negative bacteria in pink to red (Prescott and Harley 2002).

Catalase test

Dropped culture aged 24 h on a glass object, then dropped two drops of 3% H₂O₂. A positive catalase reaction was indicated by the formation of bubbles, which means the formation of oxygen by the catalase enzyme found in bacteria in the culture. LAB included catalase-negative bacteria, so it did not produce air bubbles (Melia et al. 2019).

Fermentation type test

One milliliter of LAB strain was inoculated into 9 mL of MRS Broth in a test tube, then inserted the Durham tube in an inverted position. After that, it was incubated for 48 h and observed by the presence or absence of air bubbles in the Durham tube (Melia et al. 2019).

Acid resistance test

The acid resistance test was carried out according to the method of Islam et al. (2021), in which 1 mL of bacterial culture was inoculated on 9 mL MRS broth media and then incubated at 37°C for 24 h. Then 1 mL of bacterial culture was taken from MRS broth and inoculated into a test tube containing 9 mL of MRS broth solution at pH 3 (the addition of 5N HCl adjusted pH), then incubated for 90 min. Subsequently, the dilution was carried out up to 10⁻⁶. Then it was planted using the spread method into MRS agar media, incubated at 37°C for 48 h, and counted the number of bacterial colonies that could survive using the Colony Forming Unit (CFU) plate count method. Isolates of LAB-fermented green tea with a percentage of resistance above 40% were declared to survive at pH 2 (Chua et al. 2020) and could be continued for the bile salt resistance test.

$$\frac{\text{CFU}}{\text{mL}} = \text{CN} \times \frac{1}{\text{DF}} \times \frac{1}{\text{Sample weight}}$$

Calculation of the viability of LAB is calculated by the formula:

Colony reduction (%):

$$\frac{\text{TC of LAB} - \text{TC of pH}}{\text{Total control LAB colonies}} \times 100\%$$

Where:

CFU/mL : Colony forming unit/milliliter
CN : Colony number
DF : Dilution factor
TC : Total colony

Viability (%) = 100 – colony reduction (%)

Bile salt resistance test

The resistance test to bile salts was carried out according to the method of Islam et al. (2021) with modification. Bacterial culture of 1 mL was inoculated on 9 mL MRS broth media and then incubated at 37°C for 24 h. Furthermore, 1 mL of bacterial culture from MRS broth was taken into a test tube containing 9 mL of MRS broth solution without oxgall setting (control) on MRS broth 0.3% oxgall setting and incubated for 24 h. After that, the culture of 0.3% oxgall curing and without control were diluted up to 10⁻⁶, then planted on MRS agar with the spread method, and then incubated at 37°C for 48 h. Count the number of bacteria that can survive using the cup count method with CFU. The comparison of the number of cells before and after incubation will be expressed in terms of viability (%). The higher the viability percentage produced, the more resistant the bacteria to bile salts. Isolates of LAB-fermented green tea with a percentage of resistance above 20% were declared to survive the influence of bile salts (Chua et al. 2020) so that continued for the antibacterial activity test.

Calculation of the total colonies that grew using the formula

$$\frac{\text{CFU}}{\text{mL}} = \text{CN} \times \frac{1}{\text{DF}} \times \frac{1}{\text{Sample weight}}$$

Calculation of the viability of LAB is calculated by the formula:

Colony reduction (%):

$$\frac{\text{TC LAB colonies} - \text{TC oxgall}}{\text{TC LAB colonies}} \times 100\%$$

Where:

CFU/mL : Colony forming unit/milliliter
CN : Colony number
DF : Dilution factor
TC : Total Colony

Viability (%) = 100 – colony reduction (%)

Antibacterial activity test

Antibacterial test of LAB isolate supernatant was carried out on *Escherichia coli* Q157:H7, *Staphylococcus aureus* ATCC25923, and *Listeria monocytogenes* CFSAN004330 test bacteria. LAB cultures were grown in

MRS broth for 18-22 h. Then the supernatant was separated from the cells by centrifugation at 10,700 rpm (rpm as rotation per minute) for 5 min at 37°C. Twenty milliliters of nutrient agar (NA) was poured and inoculated with 0.2% (v/v media) of the test bacteria. After it hardened, a well with a diameter of 6 mm was made. Then 50 µL of LAB supernatant was filled into each well. After that, it was incubated at 37°C anaerobically for 24 h, and the diameter of the inhibition zone was measured. The treatment was carried out three times (Thielmann et al. 2019).

Antibiotic activity test

The sensitivity test of LAB isolates to antibiotics was carried out by disc diffusion method on MRS agar. LAB cultures were grown in MRS broth and incubated at 37°C in an anaerobic jar in an incubator for 18-22 h. Furthermore, by spreading method, 100 µL of LAB culture was planted on MRS agar. A well with a diameter of 6 mm was made and then filled with 50 µL of antibiotics. Then it was incubated at 37°C for 48 h. The inhibition zone formed was measured and analyzed. The sensitivity pattern was tested using several antibiotics, namely kanamycin, and ampicillin. The treatment was carried out thrice (Halder et al. 2017).

Identification LAB

Genome DNA isolation

Ten milliliters of bacterial liquid cultures were harvested in 1.5 mL microtubes by centrifugation. The supernatant was discarded so that a cell pellet was in the microtube. Into a microtube containing pellets, 200 µL of lysis solution was added and then vortexed for 10 sec. Then 20 µL of proteinase K was added to the microtube and vortexed until the bacterial cell suspension became homogeneous. Microtubes were incubated at 56°C for 30 min. Every 10 min, the microtubes were vortexed for 5 sec. Twenty microliters of RNase solution were added to the microtube and then vortexed for 5 sec. The microtubes were then incubated at room temperature for 10 min. Four hundred microliters of cold 50% ethanol were added to the microtube and then vortexed for 5 sec. The solution in the tube was then transferred to the purification column, which was placed in the collection tube, then centrifuged at a speed of 10.000 rpm for 1 min. The collection tube, which already contained the sample solution, was discarded, while the purification column was transferred to a new collection tube. Wash buffer I of 500 µL was added to the purification column, then centrifuged at a speed of 10.000 rpm for 1 min. The solution in the collection tube was discarded, then the purification column was placed again in the collection tube. Wash buffer II of 500 µL was added to the purification column, then centrifuged at 10.000 rpm for 3 min. The collection tube was discarded, while the purification column was transferred to a new sterile 1.5 mL microtube. Elution buffer of as much as 50 µL was added right in the middle of the purification column, incubated at room temperature for 2 min, then centrifuged at a speed of 15.000 rpm for 1 min. The purification column was discarded, and the DNA solution microtube was stored at 20°C (Tiwari et al. 2017).

Genome DNA electrophoresis

The concentration of agarose gel used was 1%. Agarose powder was weighed as much as 0.5 g, put into a school bottle, and added 0.5 x TBE buffer as much as 50 mL. The bottle was heated in a microwave on medium heat for 3 min until the agarose was dissolved entirely and the solution was liquid and transparent in color. Then 5 µL of ethidium bromide was added to the bottle's inner wall slightly close to the agarose solution, then homogenized. The gel tray and comb were mounted on the agarose mold according to the number of samples used. Then the agar solution was poured into the gel tray, then wait for the agarose gel to solidify for about 20-30 min. The composition of the electrophoretic cocktail was prepared with a total volume of 10 µL consisting of genomic DNA, 1 µL 10 x BPB, and 7 µL 1 x TE.

Similarly, the cocktail composition for the DNA marker (50 ng/µL). After the agarose gel had solidified, the comb was removed first. Then the gel tray was also removed from the mold. Install the electrophoresis set, place the gel tray in the electrophoresis chamber, and add 0.5 x TBE buffer until the gel surface was immersed. Load the sample into the well carefully, starting from the leftmost for the DNA marker, followed by the genomic DNA sample, one sample in one well. Closed the chamber with the lid and then turned on the electrophoresis machine. Set the power supply using a voltage of 100 volts and a time of 30 min, and then run it. When finished, open the chamber lid, take the gel tray, and place it in the tray. The chamber was separated from the power supply, then the buffer in the chamber was transferred to the bottle provided (Psifidi et al. 2015).

Genome DNA quantification

The UV-Vis Spectrophotometer Biodrop machine was turned on by pressing the power button, then switching on the monitor screen, selecting the "Life Sciences" menu, then "Nucleotides," then "DNA." Selected the pathlength detector setting "0.5 mm lite", then the concentration unit "ng/µL," set the sample name/code, and activated the internal storage function. Cleaned the detector using tissue Kimwipes, then did the blanking first. Pipette 1 µL of solvent and place the tip of the white tip right between the detector wires on the Biodrop. Then pressed the gray cuvette icon on the monitor screen and waited until the number "0.00" appeared. The detector was cleaned again using tissue Kimwipes. Next, 1 µL of the DNA sample was pipette, pressed the orange cuvette symbol on the monitor screen, and waited until the obtained measurement numbers appeared.

16S rRNA gene amplification

Prepared a sterile 0.2 mL tube, placed it in a container filled with ice, then added forward primer 16S rRNA_F (10 ng/µL) and reverse 16S rRNA_R (10 ng/µL) each 2 µL to the bottom of the tube. A 2 µL of genomic DNA (concentration 10 ng/µL) was added to the solution in the tube. A 25 µL of KOD Blue Mastermix was added to the solution in the tube. A 19 µL of nuclease-free water was added to the solution in the tube.

Table 1. PCR reaction program

Stages temperature (°C)	Time	Cycle
Pre denaturation	95	2 minutes
Denaturation	95	45 seconds
Annealing	56	45 seconds
Extension	72	1 minute
Final extension	72	5 minutes
Cooling	8	pause

PCR ran at stages temperature and time above. The solution was then homogenized. The total reaction volume was 50 μ L. Make sure the tube had labeled, and the outside of the tube was dried. Placed the tube into the PCR machine and ran the machine according to the 16S rRNA primer program, as shown in Table 1.

After the PCR cycle was complete, the PCR reaction cocktail was electrophoresed on 1 % agarose gel. The far left well was filled with 2 μ L of 1 kb marker, then the next well was filled with 5 μ L of each PCR reaction cocktail. The tube containing the PCR reaction cocktail was then stored at -20°C. After the electrophoresis was completed, the agarose gel was visualized with the gel-doc system.

Sequencing

The PCR product of the 16S rRNA gene of bacterial samples was sequenced in two directions (bi-directional) using the Sanger sequencing method. Sequencing was performed at a sequencing service provider company, 1st Base, Singapore.

Bioinformatics analysis

The two-way sequenced electropherogram for each sample was edited and contiguous using the SeqMan™ application. The base sequence of the 16S rRNA gene for each bacterial sample was then BLAST on the NCBI website (Zhang et al. 2000). From the BLAST results, 20 bacterial sample sequence data were selected in the genebank, which was then used for alignment, phylogenetic tree construction, and genetic distance determination using the MEGA X program (Kumar et al. 2018). Alignment was performed using the Clustal W algorithm. The phylogenetic tree was constructed using the Neighbor-Joining method (Saitou and Nei 1987), and the evolutionary distance was analyzed using the Kimura 2-parameter method (Kimura 1980). The bootstrapping value used was 1,000 (Felsenstein 1985). Genetic distances were analyzed using the pairwise distances method.

RESULTS AND DISCUSSION

Green tea fermentation

The result was carried out on 6 samples of green tea, which were dried in a dehydrator at low temperatures, fermented green tea leaves were dry and flavorful. Sample 1 was 24 h fermented green tea with forest honey; sample 2 was 48 h fermented green tea with forest honey; sample 3

was 72 h fermented green tea with forest honey; sample 4 was 24 h fermented green tea with palm sugar; sample 5 was 48 h fermented green tea with palm sugar; sample 6 was 72 h fermented green tea with palm sugar and sample 7 was non-fermented green tea.

Bacterial isolation in fermented green tea

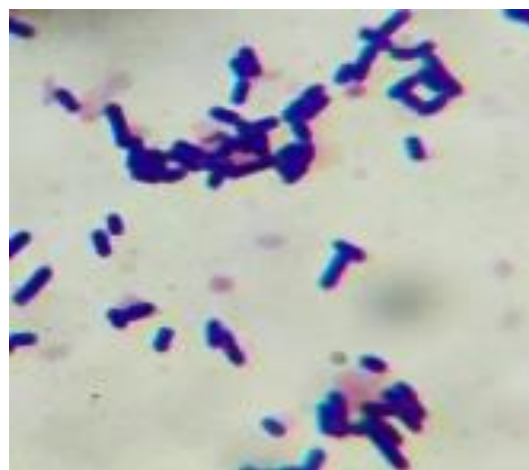
The formation of round colonies that LAB characterizes could be observed from the samples at dilutions of 10^{-6} and 10^{-7} . The macroscopic observations (color, shape, and size) of LAB found creamy-white colonies and convex surfaces with smooth edges on MRS agar media. This following the research of (Melia 2019; Syukur et al. 2022) stated that LAB colonies on MRS agar media were white-beige. LAB isolates were continued for the streaking process. The streaking process aimed to separate between colonies of microorganisms using a tool such as a long wire with a circular end (loop) or an ose needle to obtain pure cultures. Pure cultures were required to study the biochemical and morphological properties of the culture to determine the approximate number of viable bacteria in fluids or specimens (Harrigan 2014).

LAB morphology

Gram staining

The results showed that the Gram staining of the 14 plates was positive by showing the bacteria with blue-purple color on microscope observation with 1000 times magnification (Figure 2).

A Gram staining test was carried out to determine whether bacteria were Gram-positive or negative, which was indicated by the bacteria absorption of the color of the reagent. Gram-positive bacteria, if bacteria absorbed the purple crystal violet reagent, while Gram-negative bacteria if bacteria absorbed the red color of the safranin reagent (Isolauri et al. 2004). LAB was a facultative anaerobic bacterium, Gram-positive, spherical or rod-shaped, did not produce spores but produced a lactic acid which was the main product of carbohydrate fermentation (glucose, fructose, and sucrose) (Melia 2019; Ramadhanti 2021).

**Figure 2.** Gram stain results with 1000 times magnification

Catalase test

The isolate was added 1-2 drops of 3% hydrogen peroxide, left, and observed whether air bubbles formed. LAB included catalase-negative bacteria, so it did not produce air bubbles. Of the 14 LAB isolates, no air bubbles were found in the catalase test, which indicated that catalase was negative. This happens because LAB did not produce the enzyme catalase, which could convert hydrogen peroxide into water and oxygen. (König and Fröhlich 2017) stated that LAB could not produce the catalase enzyme that breaks down dihydroxy hydrogen peroxide into oxides. All isolates showed no formation of O₂ gas bubbles after the drops of H₂O₂, which indicated negative catalase. LAB included catalase-negative bacteria, so it did not produce air bubbles (Juliyarsi et al. 2018).

Fermentation type test

The fermentative test with Durham tubes with a (+) sign indicated that the fermentation was hetero-fermentative, which was indicated by the formation of air bubbles in the Durham tube. The sign (-) indicated that the fermentation was homofermentative, indicated by the absence of air bubbles in the Durham tube. The fermentative type test was conducted to classify LAB into homofermentative or heterofermentative groups. In all samples, the fermentation type was homofermentative, indicating that there was only lactic acid.

Acid resistance test

The acid resistance test result with pH 3 (the addition of 5 N HCl adjusted pH) can be seen in the following Table 2. Sample 1.1 was first colony from 24 h fermented green tea with forest honey; sample 1.2 was second colony from 24 h fermented green tea with forest honey; sample 2.1 was first colony from 48 h fermented green tea with forest honey;

sample 2.2 was second colony from 48 h fermented green tea with forest honey; sample 3.1 was first colony from 72 h fermented green tea with forest honey; sample 3.2 was second colony from 72 h fermented green tea with forest honey; sample 4.1 was first colony from 24 h fermented green tea with palm sugar; sample 4.2 was second colony from 24 h fermented green tea with palm sugar; sample 5.1 was first colony from 48 h fermented green tea with palm sugar; sample 5.2 was second colony from 48 h fermented green tea with palm sugar; sample 6.1 was first colony from 72 h fermented green tea with palm sugar; sample 6.2 was second colony from 48 h fermented green tea with palm sugar; sample 7.1 was first colony from green tea, and sample 7.2 was second colony from green tea. The results of the acid resistance test of lactic acid bacteria from fermented green tea leas sample as the following Figure 3.

Table 2. Acid resistance of LAB isolate

Samples	K	pH 3	Viability (%)
1.1	68	66	97.06
1.2	69	186	37.09
2.1	86	41	47.67
2.2	43	141	30.49
3.1	81	43	53.08
4.1	81	79	97.53
4.2	198	173	87.37
5.1	40	52	76.92
5.2	48	86	55.81
6.1	52	81	64.20
6.2	58	81	71.60
7.1	117	68	58.12
7.2	81	66	81.48

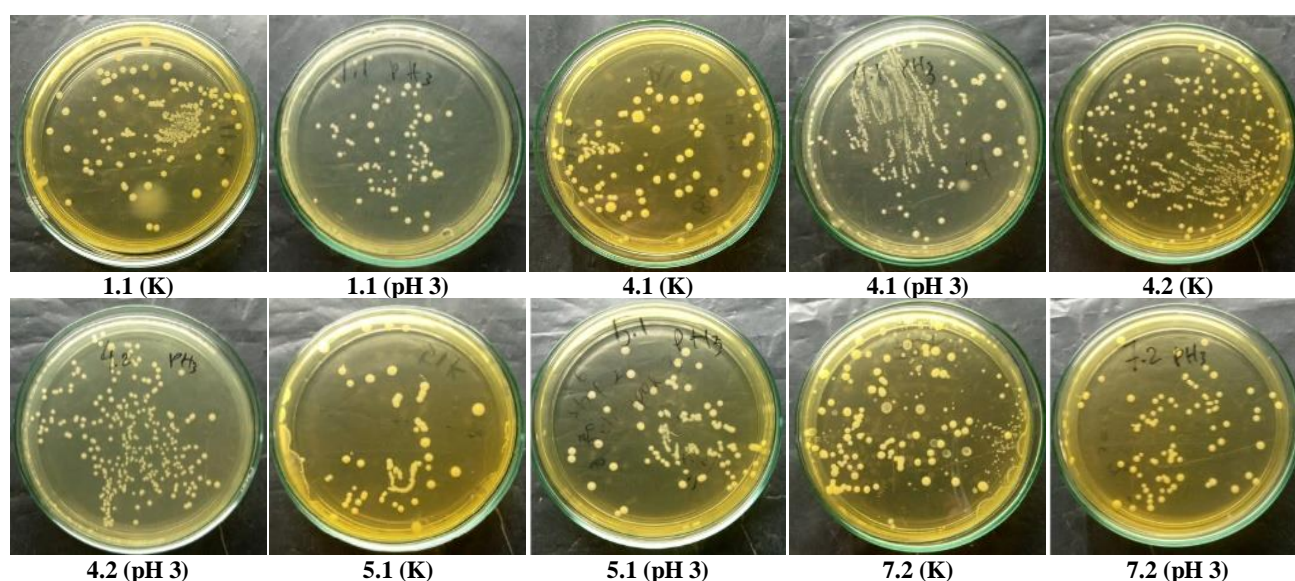


Figure 3. Acid resistance test result

The best result showed as bold number on Table 2. High viability percentage in the samples with code 1.1 (97.06%); 4.1 (97.53%); 4.2 (87.37%); 5.1 (76.92%), and 7.2 (81.48%) that continued to bile salt resistance test with Oxgall 0.3%. Fan et al. (2017) explained that LAB had different acid tolerances due to differences in the relative permeability of each LAB species. The resistance to gastric acid was one of the essential requirements for an isolate called a probiotic because if LAB isolates enter the human digestive tract, it must be able to survive at the pH of gastric acid. Based on research by Cui et al. (2014), several LAB strains were isolated from several sources. LAB, which had probiotic potential, resisted low pH with viability ranging from 80%.

The bacterial population decrease is caused by the influence of hydrochloric acid that affects cell biomolecules, DNA, proteins, and fatty acids at low pH (pH 3). Probiotic LAB is capable of traversing the human gastrointestinal tract, as well as the saliva (pH 6.5-7.5), upper stomach (pH 4.0-6.5), lower stomach (pH 1.5-4.0), and intestinal (pH 4.0-7.0) (Fallingborg 1999; Sanders 2003). As a consequence, it gives the product beneficial health effects. This will enable the bacteria to thrive, proliferate, and produce the essential positive effects in the digestive tract, recreating the lactic acid microflora responsible for the body's immune system. In addition to surviving acidic conditions in the stomach, probiotics must also be capable of living in intestine bile salts to exert their therapeutic effects (Mulaw et al. 2019; Yang et al. 2020), because bile salts and pancreatin can contribute to adverse conditions in the small intestine.

Bile salt resistance test

The viability of the bile salt resistance test is shown in Table 3.

The best result showed as bold number. High viability percentage of the samples with code **1.1** (93.98%) and **4.1** (86.70%), continued to the antibacterial activity test. Both bacteria resulted from different fermented green tea with the same fermentation time (24 h). The higher the viability of bacteria obtained, the resistance of probiotic bacteria to bile salts was also high. The bile acid content in the liver, which was created from cholesterol and produced from the gall bladder into the duodenum, should also survive the probiotics. The isolates' capacity to endure in bile salts facilitated bacteria's colonization and metabolic activity in the host's small intestine. Human bile concentrations range from 0.3% to 0.5%.

LAB was said to be a probiotic if it could survive on bile salts in the digestive tract because it was a Gram-positive bacterium with thick peptidoglycan, so bile salts did not easily damage it. LAB was said to be a probiotic if it could survive when it entered the upper part of the intestinal tract where bile salts were secreted in the intestine (Talib et al. 2019). The resistance of LAB to bile salts was related to the enzyme Bile Salt Hydrolase (BSH), which helped to hydrolyze conjugated bile salts, thereby reducing the toxic effect on cells (Harun et al. 2020).

Antibacterial activity

The antibacterial activity of fermented green tea against pathogenic bacteria (*L. monocytogenes* CFSAN004330 (Gram-positive bacteria), *E. coli* Q157:H7 (Gram-negative bacteria), and *S. aureus* ATCC25923 (Gram-positive bacteria)) had been done as Figure 4. The inhibition zone diameter of plates containing kanamycin (antibiotic), ampicillin (antibiotic), LAB isolates 1.1, and LAB isolate 4.1 against pathogen bacteria *E. coli* Q157:H7, *S. aureus* ATCC25923, and *L. monocytogenes* CFSAN004330. For details, see the Table 4.

The inhibition zone diameter from kanamycin and ampicillin as antibiotics had no different results with LAB isolate 1.1 and 4.1, which means they all had inhibition activity well. Both 1.1 and 4.1 were different LAB isolates with same fermentation time (24 h). 1.1 was LAB isolate from fermented green tea with forest honey, and 4.1 was LAB isolate from fermented green tea with palm sugar. The purpose of using kanamycin and ampicillin antibiotics was because the pathogenic test bacteria used in this study belonged to the gram-positive and negative test bacteria, and the antibiotic as a positive control was able to play an active role against it. The diameter of the inhibition zone against pathogenic bacteria was 0-3 mm clear zone with low antimicrobial activity, >3-6 mm moderate, and >6 mm high antimicrobial activity (Boukhris et al. 2020). All antibacterial activity tests from LAB isolate 1.1 and 4.1 against the pathogen bacteria (*E. coli* Q157:H7, *S. aureus* ATCC25923, and *L. monocytogenes* CFSAN004330) were categorized as having high antibacterial activity. Much of this antimicrobial effect is due to the formation of lactic and acetic acids and the resulting decrease in pH. In addition, lactic acid bacteria also produce other inhibitory compounds such as hydrogen peroxide, diacetyl, carbon dioxide, reuterin, and bacteriocin (De Vuyst and Vandamme 1994).

Table 3. Bile salt resistance of LAB isolate

Isolate of LAB	K1	K2	K3	K average	OX ₁	OX ₂	OX ₃	OX average	Viability (%)
1.1	188	191	169	182.67	165	178	172	171.67	93.98
4.1	155	167	141	155.33	131	148	125	134.67	86.70
4.2	211	205	216	210.67	189	155	156	166.67	79.11
5.1	168	124	179	157	121	119	54	98	62.42
7.2	128	134	131	131	121	106	112	113	86.25

Table 4. Antibacterial activity of LAB isolates against *Escherichia coli* Q157:H7, *Staphylococcus aureus* ATCC25923, and *L. monocytogenes* CFSAN004330

Sample	IZD <i>E. coli</i> Q157:H7 (mm)			IZD <i>L. monocytogenes</i> CFSAN004330 (mm)			IZD <i>S. aureus</i> ATCC25923 (mm)		
	1	2	3	1	2	3	1	2	3
Kanamycin	12	11	13	10	7	10	14	12	13
Ampicillin	-	9	9	10	9	8	10	8	9
LAB Isolate 1.1	8	12	7	10	9	8	11	10	10
LAB Isolate 4.1	9	10	9	9	8	7	12	10	11

Note: IZD: Inhibition zone diameter; LAB: Lactic acid bacteria

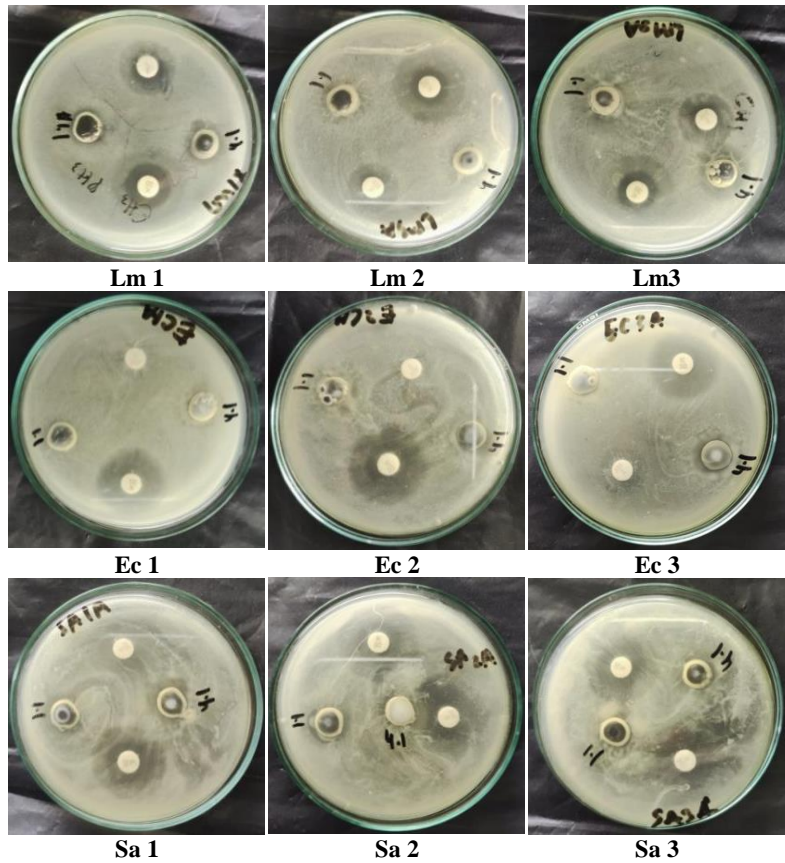


Figure 4. Antimicrobial activity test of fermented green tea against bacteria Lm: *L. monocytogenes* CFSAN004330, Ec: *E. coli* Q157:H7, Sa: *S. aureus* ATCC25923, and 1,2,3: repetition

Some lactic acid bacteria have antimicrobial activity against pathogenic bacteria and also have probiotic potential. Organic acids and hydrogen peroxide produced by lactobacilli can inhibit the growth of Gram-positive and Gram-negative bacteria, while bacteriocin has more effect on Gram-positive bacteria (Vasiee et al. 2014). Lactic acid bacteria produce lactic acid, the main glucose fermentation product (Arena et al. 2017).

Visualization of 16S rRNA gene amplification results for LAB isolates is shown in Figure 5.

Based on the visualization of the PCR results, thick and light bands were obtained following the estimated target size of 1,498 bp (red band). However, other thin bands of various sizes also appeared apart from the target band. These thin bands' appearance was caused by the presence of the multicopy 16S rRNA gene in the bacterial sample genome, resulting in primer attachment to other regions of the genome.

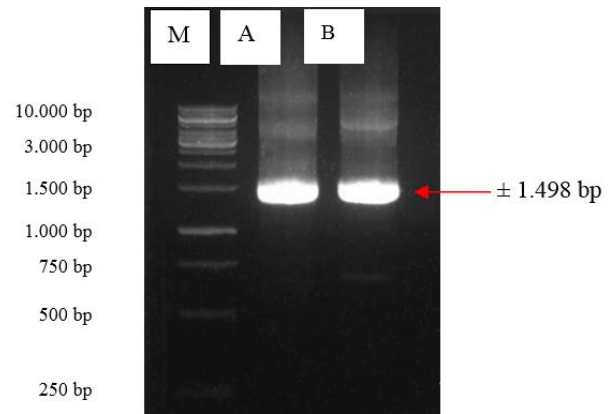


Figure 5. Visualization of 16S rRNA gene amplification results for LAB isolates. Note: M: Marker 1kb gene ruler (ThermoScientific, USA), A: PCR product of 16S rRNA gene LAB isolate 1.1, B: PCR product of 16S rRNA gene LAB isolate 4.1

LAB identification

Both LAB isolates 1.1, and 4.1 consist of different *Lactobacillus* species. LAB isolate 1.1 had the closest position in the form of the same branch as the bacterium *Lactobacillus plantarum* strain Y-1. Meanwhile, LAB isolate 4.1 had the closest level of kinship with the bacteria *Lacticaseibacillus paracasei* strain HBUAS62903. In LAB isolate 1.1, the results of the sequencing of the forward and reverse primary readings were not contiguous, so the sequence editing was done separately. The sequencing electropherogram consisted of non-single peaks. According to the 1st Base, this happened because of two possibilities. That was the first because the bacterial isolate used in the genomic DNA isolation process was not a single and pure isolate. The second possibility was the presence of multicopy target genes in the bacterial genome. The PCR product from the bacterial genomic DNA LAB Isolate 1.1 also appeared to produce bands other than the target band. This means that the primers used have binding sites in other regions of the bacterial genome.

The primary forward reading results obtained were 596 bp in size. The first 20 bp of the base at the 5' end (upstream) and the last 123 bp of the base at the 3' end (downstream) were cut because the electropherogram's peaks were unclear and overlapped. Subsequently, editing was carried out so that the total length of the sequence from the reading of the 16S rRNA forward primer gene for bacteria LAB isolate 1.1 was known to be 449 bp in size. 16S rRNA sequencing of LAB isolate 1.1 was forward primer reading (449 bp long base sequence) and reverse primer reading (890 bp long base sequence). Based on the BLAST results, the cover query value obtained from 100 samples of comparison bacteria was 100%. Meanwhile, the percent identity value obtained was in the range of 99.10-99.21%. A total of 20 comparison sequence data were selected for alignment, phylogenetic tree construction, and genetic distance analysis.

The method used for constructing the phylogenetic tree is the Neighbor-Joining method, with a bootstrap value of 1,000. The evolutionary distance was analyzed using the Kimura 2-parameter method (Horiike 2016). The phylogenetic tree of the LAB Isolate 1.1 sequence data (Figure 6).

Two groups were obtained called group A and group B. Group A consisted of 11 bacteria, while Group B consisted of 10 bacteria. Bacteria Isolate 1.1 was in Group A. Based on the position on the phylogenetic tree, LAB Isolate 1.1 had the closest position in the form of the same branch as the bacterium *Lactobacillus plantarum* strain Y-1 (LT853606.1). This meant that LAB Isolate 1.1 had the closest relationship with the bacteria *Lactobacillus plantarum* strain Y-1 (Evanovich et al. 2019).

Lactobacillus plantarum was a facultative anaerobic lactic acid bacterium widely distributed and commonly found in fermented foods and the human digestive tract. Facultative anaerobes were the ability of bacteria without oxygen to ferment and convert sugar into lactic acid. Foods

like dairy products (fermented milk and cheese), vegetables (pickles, table olives, sauerkraut, etc.), and sausages could all be fermented using these bacteria. It has been demonstrated that taking *L. plantarum* while taking antibiotics helps lessen some gastrointestinal problems (Melgar-Lalanne et al. 2012).

Sequencing results from the 16S rRNA-F forward primer measurement reading was 623 bp, and the reading of the reverse primer 16S rRNA-R measurement was 1,033 bp. Editing was done by cutting areas that had crumpled, indistinct, and overlapping peaks at the 5' and 3' ends of each primary reading. In the 16S rRNA-F primer, 19 bp at the 5' end and 44 bp at the 3' end were cut. Meanwhile, in the 16S rRNA-R primer, 15 bp of the base was cut at the 3' end and 47 bp at the 5' end. The total length of the 16S rRNA gene sequence identified from the bacterial sample LAB isolate 4.1 was 1,451 bp. Bacterial 16S rRNA sequence LAB isolates 4.1 (base sequence 1,451 bp).

Alignment with Clustal W and phylogenetic tree construction was done using the MEGA X application. Based on the alignment with Clustal W, 5 variations of bacterial sequence data LAB isolate 4.1 compared to 20 other bacteria. The phylogenetic tree construction was formed using the Neighbor-Joining method with a bootstrap value of 1,000. The evolutionary distance was analyzed using the Kimura 2-parameter method (Horiike 2016). The phylogenetic tree oLAB isolate 4.1 sequence data obtained was as follows (Figure 7).

Two groups were obtained called group A and group B. Group A consisted of 14 bacteria, while group B consisted of 7 bacteria. LAB isolate 4.1 was in group A. Based on the position on the phylogenetic tree, LAB isolates 4.1 was in the same branch as the bacteria *Lacticaseibacillus paracasei* strain HBUAS62903 (ON130253.1). Based on the results of genetic distance analysis, the comparison value of the 16S rRNA gene sequence of bacteria LAB isolate 4.1 with 20 comparison samples, the lowest was 0.0020704 in sample number 8. Sample number 8 was the bacteria *Lacticaseibacillus paracasei* strain HBUAS62903 (ON130253.1), so it could be concluded that the LAB isolate 4.1 had the closest level of kinship with the bacteria *Lacticaseibacillus paracasei* strain HBUAS62903 (Torres et al. 2022).

Lacticaseibacillus paracasei was a species of gram-positive and homofermentative lactic acid bacteria commonly used in dairy product fermentation and as a probiotic culture. *Lc. paracasei* was a bacterium that operates by means of commensalism. Commensalism is a relationship between two organisms in which one benefits and the other was neither harmed nor benefited. *Lc. paracasei* was commonly found in many human habitats such as the intestinal tract and human mouth as well as sewage, silage, and dairy products. Its name included the morphology, the bacterium was rod-shaped (bacillus shape) 2.0 to 4.0 µm wide, and 0.8 to 1.0 µm long (Orlando et al. 2012; Kiouisi 2022).

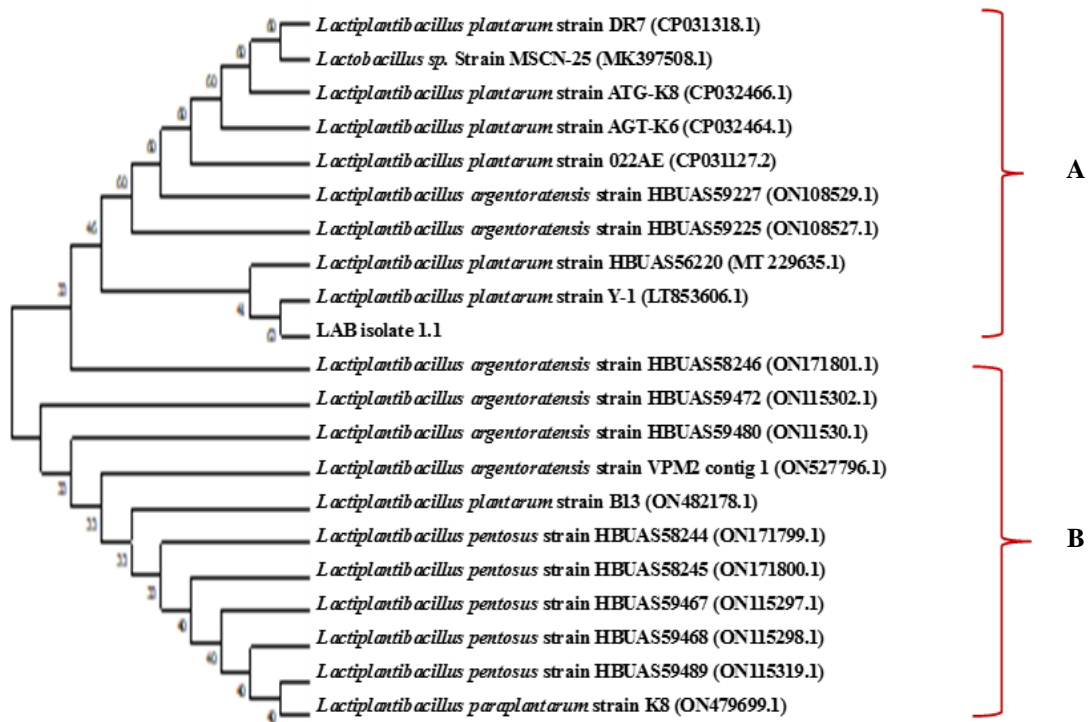


Figure 6. Phylogenetic tree from *Lactobacillus plantarum* strain Y-1 LT853606.1 (LAB isolate 1.1)

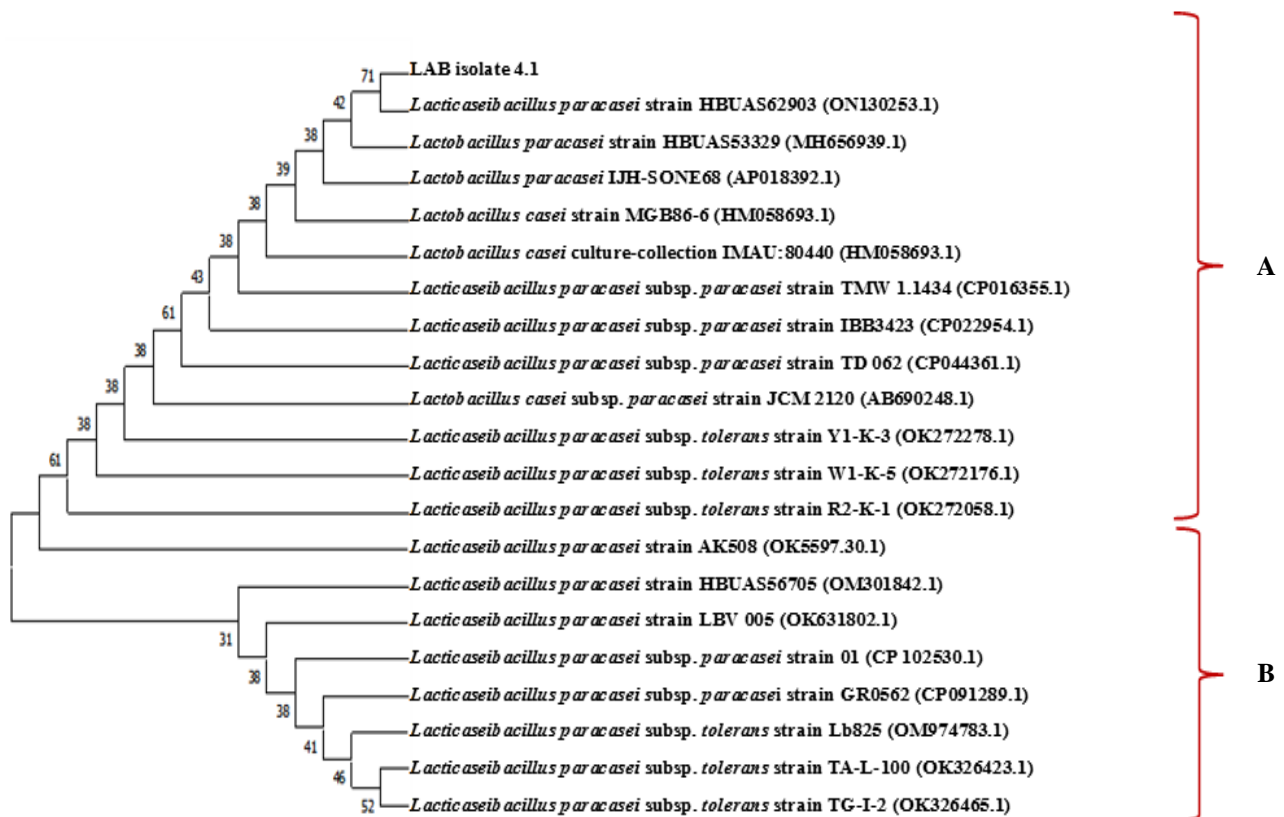


Figure 7. Phylogenetic tree from *Lacticaseibacillus paracasei* strain HBUAS62903 (LAB isolate 4.1)

In conclusion, the results identified two lactic acid bacteria isolated from fermented green tea from Sidamanik. *Lactobacillus plantarum* strain Y-1 was isolated from fermented green tea with forest honey and *Lacticaseibacillus paracasei* strain HBUAS62903 (ON130253.1) was isolated from fermented green tea with palm sugar. The two bacteria were obtained from the best fermentation results, namely 24-hour fermentation which had been characterized and tested for morphological characteristics and biochemistry tests (gram staining, catalase tests, fermentation type tests), resistance to acids and bile salts, and also antibacterial tests against three pathogenic bacteria (*E. coli* Q157:H7, *S. aureus* ATCC25923, and *L. monocytogenes* CFSAN004330) gave the best results compared to fermented green tea samples which were fermented for 48 h and 72 h. Further research is expected to be tested preclinically on test animals and examined at the molecular level whether fermented green tea containing *Lactobacillus plantarum* strain Y-1 and *Lacticaseibacillus paracasei* are safe for consumption and provides health benefits so that later it can be developed for human treatment.

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